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These studies are designed to determine whether ethanol antagonizes the ability of the skeleton to adapt to increased mechanical usage. Ethanol reversibly alters the biophysical properties of cell membranes. The overall hypothesis to be tested in adult rats is that these membrane changes disrupt essential cell signaling pathways for one or more cytokines, growth factors and polypeptide hormones that regulate bone modeling and remodeling. This report summarizes our progress from 01 September 2000 to 31 August 2001. During Year 3 of the award we have continued analysis of experiments performed in Year 2 related to Tasks 7 and 8. Additional experiments were performed to accomplish Task 6. The new studies are directed toward determination of the effects of ethanol on: the skeletal response to unloading (Task 6), skeletal adaptation to treadmill running (Task 7), and PTH-induced increases in mRNA levels for bone matrix proteins (Task 8). The results to date strongly support our hypothesis that alcohol abuse contributes to skeletal injuries suffered during the course of rigorous physical training activities.

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(4) INTRODUCTION

Chronic alcohol abuse is an important risk factor for osteoporosis (1-4). Alcohol also inhibits bone formation in experimental animals and disrupts cell signaling in cultured osteoblasts (5-12). The ultimate goal of this research is to identify the cellular and molecular mechanisms responsible for mediating ethanol's dose- and time-dependent actions on bone turnover, mass, architecture, and strength. It is well established that ethanol reversibly alters the biophysical properties of cell membranes and in doing so disturbs normal membrane function. The proposed studies in young adult rats will test our working hypothesis that these membrane changes disrupt essential cell signaling pathways for one or more bone cells "coupling" factors and/or polypeptide hormones that regulate bone modeling and remodeling. These changes are postulated to lead to the bone loss associated with chronic alcohol abuse. If our hypothesis is correct, then ethanol antagonizes the ability of the skeleton to respond to weight bearing because the signal transduction pathways for mechanical signals require peptide signaling molecules as intermediates. This latter effect of ethanol to reduce the ability of the skeleton to adapt to increased mechanical stress would be especially detrimental during rigorous military training.

(5) BODY

Introduction

Progress relevant to Tasks 1-5 as well as Task 8 were reported in the Year 1 progress report. Data analysis was continued in Year 2 and abstracts and manuscripts describing this work were submitted in Year 2 (see item 7, Reportable Outcomes and item 10, Appendices for an update). Additional work directed toward accomplishing Tasks 6 and 7 were performed in Year 3 and will be described in this report.

We will discuss the progress for each Task separately. Appendix 1 lists experiment number, title, and Task(s).

Task 6

The goal of this Task was to determine the effects of ethanol on skeletal adaptation to normal weight bearing following unloading. We performed a preliminary study to validate the method in our laboratory. As anticipated, hindlimb unloading for two weeks resulted in decreased bone formation and bone loss (Table 1). We are in the process of a second study to determine the combined effects of ethanol and hind limb unloading on bone metabolism in 6-month-old male rats. We have performed the two week long unloading portion of the study and have analyzed cortical bone histomorphometry. Analysis of the cancellous bone histomorphometry will be completed in Year 4.

The cortical bone data is shown in Table 2. Hindlimb unloading and ethanol each resulted in decreased bone formation. Two-way ANOVA demonstrated that in combination unloading and ethanol have additive inhibitory effects.

Task 7

The goal of this Task was to determine the effects of ethanol on the skeletal adaptation to treadmill running. We have completed a 4-month-long animal study and have performed histomorphometry and biochemical measurements. We have analyzed the data and are nearing completion of a manuscript. Briefly, we have found that ethanol results in highly detrimental effects on the skeleton of exercising rats (Table 3). These findings suggest that the risk for fracture attributable to vigorous exercise is greatly enhanced by ethanol.

Task 8

Task 8 was accomplished in Year 2. However, we performed an additional 1 week study to compare the effects of pulsatile PTH, continuous infusion of PTH and alcohol on gene expression in the skeleton. Pulsatile PTH increases bone formation and bone mass, and is being investigated as a treatment for osteoporosis. Continuous PTH is a model for chronic hyperparathyroidism which causes severe bone disease. Using micro gene arrays, a technology that was not available when this proposal was submitted, we identified genes that were differentially expressed by the 3 treatments. By performing subtractive hybridization of RNA from rats treated with continuous and pulsatile PTH we hope to determine the genes responsible for the anabolic and catabolic effects of PTH. Similarly, by performing subtractive hybridization of RNA from rats treated with ethanol alone, pulsatile PTH alone and a combination of PTH and ethanol we hope to identify the genes responsible for the detrimental effects of alcohol. Using this approach, we have identified that the probable causative factor for osteitis fibrosa is over-expression of platelet derived growth factor-A (PDGF-A) (see item 7-Reportable Outcomes). This finding is relevant to this project because PDGF may also play a role in the etiology of alcohol-induced liver disease.

(6) KEY RESEARCH ACCOMPLISHMENTS

We are on target to complete all 10 of the proposed Tasks during the 4 year grant period. We have now shown that ethanol: 1) blunts the skeletal response to PTH, a key physiological regulator of mineral homeostasis; 2) increases the risk of injury during rigorous exercise by inhibiting bone remodeling and reducing bone mass; and 3) accentuates the detrimental skeletal response to disuse.

(7) REPORTABLE OUTCOMES

Turner RT: Skeletal response to alcohol. Alcohol Clin Exp Res, 24:1693-1701, 2000.

Turner RT, Kidder LS, Kennedy A, Evans GL, Sibonga JD: Moderate alcohol consumption suppresses bone turnover in adult female rats. J Bone Miner Res, 16:589-594, 2001.

Turner RT, Maran A, Lotinun S, Hefferan T, Evans GL, Zhang M, Sibonga JD: Animal models for osteoporosis. Reviews in Endocrine and Metabolic Disorders, 2:117-127, 2001.

Maran A, Zhang M, Spelsberg TC, Turner RT: The dose-response effects of ethanol on the human fetal osteoblastic cell line. J Bone Miner Res 16:270-276, 2001.

Turner RT, Evans GL, Zhang M, Sibonga JD: Effects of parathyroid hormone on bone formation in a rat model for chronic alcohol abuse. Alcohol Clin Exp Res, 25:667-671, 2001.

Turner RT, Sibonga JD: Effects of alcohol and estrogen on bone metabolism. Alcohol Research and Health, Accepted with minor revisions.

Turner RT: Skeletal adaptation to external loads to optimize mechanical properties: Fact or fiction. Current Opinion in Orthopaedics, Submitted.

Lotinun S, Turner RT: Triazolopyrimidine (trapidil) inhibits the detrimental effects of parathyroid hormone in an animal model for chronic hyperparathyroidism. Nature Medicine, Submitted.

(8) CONCLUSIONS

Our results to date strongly support our original hypothesis that chronic alcohol abuse increases the risk of injury to the skeleton during military training.

Table 1: The Effects of Hindlimb Unloading on Cancellous Bone Histomorphometry			
Measurement	Baseline	Loaded	Unloaded
BV/TV (%)	28.3±2.2	26.7±2.3	22.6±1.6 ^a
Tb.Th (µm)	71.3±3.2	72.3±3.5	65.0±3.6
Tb.N (mm ⁻¹)	3.96±0.26	3.73±0.13	3.46±0.17 ^a
LS (%)	--	8.6±1.6	0.1±0.1 ^b
MAR (µm/d)	--	0.90±0.05	0.64±0.06 ^b
BFR (%/d)	--	0.082±0.017	0.001±0.001 ^b
^a p < .05 compared to baseline; ^b p < .05 compared to loaded control. Values are mean ± SE; N=9-10 Bone volume (BV); Tissue volume (TV); Trabecular thickness (Tb.Th); Trabecular number (Tb.N); Labeled surface (LS); Mineral apposition rate (MAR); Bone formation rate (BFR).			

Table 2: Effects of ethanol and Hind Limb Unloading on Cortical Bone Histomorphometry in Six-Month-Old Rats

Measurement					ANOVA (p value)		
	Loaded Control	Loaded Ethanol	Unloaded Control	Unloaded Ethanol	Loading	Ethanol	Interaction
Cross sectional area (mm ²)	4.82±.11	5.06±.08	4.96±.10	5.03±.17	NS	NS	NS
Medullary area (mm ²)	.95±.03	1.08±.04	.97±.04	1.03±.04	NS	.02	NS
Cortical area (mm ²)	3.87±.10	3.98±.06	3.99±.08	4.00±.14	NS	NS	NS
Bone formation rate (mm ² x10 ⁻³ /d)	16±2	11±1	7±1	4±1	.0001	.02	NS
Mineral apposition rate (µm/d)	2.8±.2	2.1±.2	1.9±.2	1.4±.1	.0001	.0003	NS
Labeled perimeter (mm)	.18±.03	.12±.02	.07±.01	.05±.01	.0001	.0003	NS

Values are mean ±SE; N=10.

Table 3: The Effects of Exercise and Alcohol Consumption on Cancellous Bone Histomorphometry							
Variable	Ex-EtOH	Sham-EtOH	Ex	Sham	2-way ANOVA		
					Effect of Exercise	Effect of EtOH	Interaction
BV/TV (%)	12.69±2.25	12.48±1.80	16.62±2.12	14.71±2.63	NS	NS	NS
BFR/BS (µm ³ /µm ² /d)	.069±.012	.088±.022	.132±.016	.136±.020	NS	P = .01	NS
BFR/BV (%)	.211±.031	.272±.056	.325±.033	.383±.067	NS	P = .02	NS
BFR/TV (%)	.027±.006	.034±.011	.052±.007	.056±.013	NS	P = .02	NS
LS/BS (%)	9.31±1.36	9.80±1.73	20.66±4.61	15.64±1.78	NS	P = .01	NS
MAR (µm/d)	.727±.054	.829±.056	.797±.025	.850±.040	NS	NS	NS
Tb.Th (µm)	64.24±5.61	63.57±4.82	81.90±5.48	74.59±8.22	NS	P = .02	NS
Tb.N (No./µm)	1.83±.23	1.87±.17	1.95±.15	1.89±.21	NS	NS	NS
Tb.Sp (µm)	587.31±103.05	521.28±68.82	472.08±49.97	523.64±87.80	NS	NS	NS

All data are expressed as means ± SE; n = 9-13/group.

Bone volume, BV; Tissue volume, TV; Bone formation rate, BFR; Labeled surface, LS; Bone surface, BS; Mineral apposition rate, MAR; Trabecular thickness, Tb.Th; Trabecular number, Tb.N; Trabecular separation, Tb.Sp.

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(10) APPENDICES

Appendix 1: Summary of Experiments Performed and/or Analyzed in Year 3

Appendix 2: Turner RT: Skeletal response to alcohol. Alcohol Clin Exp Res, 24:1693-1701, 2000.

Appendix 3: Turner RT, Kidder LS, Kennedy A, Evans GL, Sibonga JD: Moderate alcohol consumption suppresses bone turnover in adult female rats. J Bone Miner Res, 16:589-594, 2001.

Appendix 4: Turner RT, Maran A, Lotinun S, Hefferan T, Evans GL, Zhang M, Sibonga JD: Animal models for osteoporosis. Reviews in Endocrine and Metabolic Disorders, 2:117-127, 2001.

Appendix 5: Maran A, Zhang M, Spelsberg TC, Turner RT: The dose-response effects of ethanol on the human fetal osteoblastic cell line. J Bone Miner Res 16:270-276, 2001.

Appendix 6: Turner RT, Evans GL, Zhang M, Sibonga JD: Effects of parathyroid hormone on bone formation in a rat model for chronic alcohol abuse. Alcohol Clin Exp Res, 25:667-671, 2001.

Appendix 7: Turner RT, Sibonga JD: Effects of alcohol and estrogen on bone metabolism. Alcohol Research and Health, In press.

Appendix 8: Turner RT: Skeletal adaptation to external loads optimizes mechanical properties: Fact or fiction. Current Opinion in Orthopaedics, In press.

Appendix 9: Lotinun S, Turner RT: Triazolopyrimidine (trapidil) inhibits the detrimental effects of parathyroid hormone in an animal model for chronic hyperparathyroidism. Nature Medicine, Submitted.

Appendix 1: Summary of Experiments Performed and/or Analyzed in Year 3

Experiment #	Title	Task Number(s)
11*	Effects of Alcohol on Skeletal Adaptation to Treadmill Running	7
15	Effects of Hindlimb Unloading on Bone Histomorphometry	6
16	Effect of Alcohol on the Skeletal Response to Hindlimb Unloading	6
17	Differential Effects of Pulsatile PTH, Continuous PTH and Alcohol on Gene Expression in Rat Bone	8
* Experiment #s 1-10, and 12-14 were reported previously.		

Skeletal Response to Alcohol

Russell T. Turner

This review briefly assesses the well-established effects of alcohol consumption on bone and mineral metabolism and addresses areas of controversy that need additional research. Alcohol consumption is a risk factor for osteoporosis based on the frequent finding of a low bone mass, decreased bone formation rate, and increased fracture incidence in alcoholics. Alcohol also has been shown to reduce bone formation in healthy humans and animals and to decrease proliferation of cultured osteoblastic cells. On the other hand, it has been difficult to demonstrate alcohol-induced bone loss and increased fracture rate in population-based studies. Indeed, most population-based studies have shown a positive association between alcohol and bone mass and no change or a decrease in fracture risk. Overall, the evidence generally supports a detrimental effect of chronic alcohol abuse on the skeleton of men and a neutral or generally beneficial effect of light to moderate alcohol consumption, especially in older women. This latter putative beneficial effect may be due to a reduction in the age-related increase in bone remodeling associated with postmenopausal bone loss. Specific areas of research are recommended to clarify the dose and sex effects of alcohol consumption and to determine cellular and molecular mechanisms of action. The goals of this proposed research emphasis are to determine the degree of risk for the range of alcohol consumption, to set guidelines of consumption compatible with maintaining bone health, and to develop appropriate countermeasures to prevent or reverse the detrimental skeletal effects of alcohol abuse.

Key Words: Skeleton, Alcohol, Bone Metabolism, Mineral Metabolism.

THIS ARTICLE WILL review the effects of alcohol on bone and mineral metabolism. It will emphasize the association between alcohol consumption and osteoporosis. Additional goals are to identify areas of controversy and suggest priorities for future research.

EFFECTS OF ALCOHOL ABUSE ON BONE TURNOVER IN HUMANS

Histological studies suggest that alcohol abuse is associated with osteopenia due in part to decreased osteoblast activity (Bikle et al., 1993; Schnitzler and Solomon, 1984). The histological evidence for decreased bone formation is supported by consistent findings of reduced serum osteocalcin, a biochemical marker of bone formation (Gonzalez-Calvin et al., 1993; Labib et al., 1989; Laitinen et al., 1991a, 1992, 1994; Nielsen et al., 1990; Rico et al., 1987). In contrast, the reported effects of alcohol abuse on histological and biochemical markers of bone resorption are contradictory, with evidence for no change as well as for decreased and increased bone resorption reported (Bikle et al., 1993, 1985; Crilly et al., 1988; Diez et al., 1994; Laitinen

et al., 1991a, 1994; Lalor et al., 1986; Schnitzler and Solomon, 1984).

There is evidence that duration of alcohol abuse is associated positively with the severity of osteopenia, which suggests that the bone loss is gradual (Harding et al., 1988; Odvina et al., 1995; Pumarino et al., 1996). A slow rate of bone loss suggests a small imbalance between bone formation and bone resorption that allows bone resorption to predominate as the principal cellular mechanism that leads to osteopenia. The conclusion of some investigators (Gonzalez-Calvin et al., 1993; Laitinen et al., 1991a, 1994) that bone remodeling is uncoupled (bone formation is decreased and bone resorption increased) is difficult to reconcile with an apparent gradual rate of bone loss. When it occurs in other medical conditions, including immobilization and glucocorticoid excess, uncoupled bone remodeling results in significant bone loss within months (Leblanc et al., 1990; Reid, 1999). However, there have been no longitudinal studies, so it cannot be excluded that alcohol-induced bone loss is punctuated with brief periods of rapid bone loss followed by prolonged intervals with little or no change in bone mass.

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THE EFFECTS OF MODERATE DRINKING ON THE HUMAN SKELETON

Long-term detrimental effects of alcoholism to reduce bone mass, although not universal, are relatively well established. In contrast, the skeletal effects of moderate alcohol consumption on bone and mineral homeostasis are

relevant to a greater number of people but are less certain. Reports of improved bone mass, especially among postmenopausal women, are intriguing but not fully understood (Feskanich et al., 1999; Laitinen et al., 1991b, 1993; Orwoll et al., 1996).

MECHANISMS OF ACTION OF ALCOHOL ON BONE METABOLISM

The mechanisms of action of alcohol on bone turnover are not understood and may include both direct and indirect actions. The indirect actions may occur secondarily to changes in calcium-regulating hormones, mineral homeostasis, and mechanical loading (due to decreased body weight). Alcoholics often become hypomagnesemic and hypocalcemic and may have hypocalciuria (Bikle et al., 1985; Diez et al., 1994; Gonzalez-Calvin et al., 1993; Laitinen et al., 1992; Lalor et al., 1986). Vitamin D metabolism also can be disturbed. In patients with cirrhosis, intestinal absorption of vitamin D is diminished and the half-life of the labeled vitamin after intravenous administration is shortened (Barragry et al., 1979). Serum 25-hydroxyvitamin D [25(OH)D] was normal or reduced in patients with alcoholic liver disease (Hepner et al., 1976). Serum levels of 1,25-dihydroxyvitamin D [1,25(OH)₂D] were normal or reduced in alcoholics, and serum parathyroid hormone levels were normal or elevated (Bikle et al., 1985; Bouillon et al., 1984). 1,25(OH)₂D can influence bone metabolism directly or via its actions to stimulate secretion of parathyroid hormone. Thus, it is possible that some of the effects of chronic alcohol abuse on bone mass are mediated by disturbances in the regulation of these important calcium-regulating hormones. However, no cause and effect relationship has been established, and abnormal calcium homeostasis is not a universal finding in alcoholics. Again, most studies have emphasized the effects of alcohol on mineral homeostasis in alcoholics; the possible effects of alcohol on light to moderate drinkers are even less clear and may differ both qualitatively and quantitatively from the effects of alcohol abuse. Another complication is that underage drinking may reduce peak skeletal mass, which creates a lower threshold for development of osteopenia later in life. However, there have been no human studies that focused on the effects of adolescent drinking on bone and mineral homeostasis.

The evidence for direct effects of alcohol on bone cell metabolism was obtained from cell culture studies. Alcohol was reported to increase indices of bone resorption in isolated osteoclasts and decrease indices of osteoblast differentiation (e.g., alkaline phosphatase activity) in osteoblast-like cells (Chavassieux et al., 1993; Cheung et al., 1995). High concentrations of alcohol inhibit the proliferation of osteosarcoma cells but do not appear to reduce cell lifespan (Klein and Carlos, 1995; Klein et al., 1996). This latter finding suggests that alcohol does not have a direct toxic effect on mature osteoblasts. It is especially interest-

ing that alcohol interacts with skeletal signaling peptides to modify the response of bone cells to these important regulatory peptides (Farley et al., 1985). Although extrapolation of cell culture data to physiological systems must be viewed with extreme caution, these observations support the hypothesis that osteoblast activity and cell signaling are disturbed by alcohol. Unfortunately, *in vitro* studies are unable to address the effects of alcohol on recruitment of and initiation of bone remodeling by osteoclasts, and there are no established *in vitro* models for studying the coupling of bone formation to bone resorption.

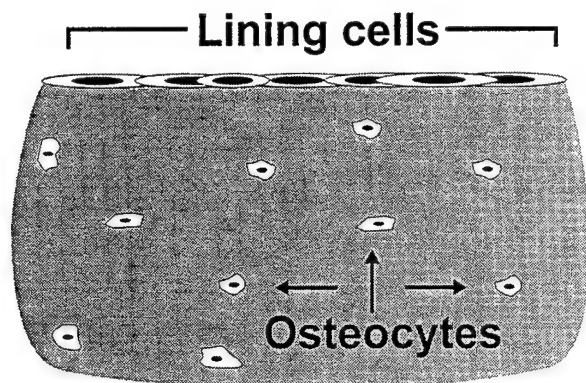
REGULATION OF BONE BALANCE IN HUMANS AND LABORATORY ANIMALS

Bone is remodeled continuously throughout life by a process that was described by Frost (1969). There is indirect evidence that bone remodeling, which is essential to maintain normal bone mass and architecture in the mature skeleton, can be disturbed by alcohol. Figure 1, which was adapted from Parfitt (1984), illustrates the bone remodeling cycle. Cancellous bone remodeling occurs when bone resorption is initiated on a previously quiescent surface. A discrete unit of bone is removed, and the resulting resorption lacuna is filled with new bone shortly thereafter. The formation phase of the bone remodeling sequence is mediated by osteoblasts that replace the osteoclasts in the resorption lacuna and theoretically can underfill, precisely fill, or overfill the lacuna, which results in a small decrease, no change, or a small increase in bone volume, respectively.

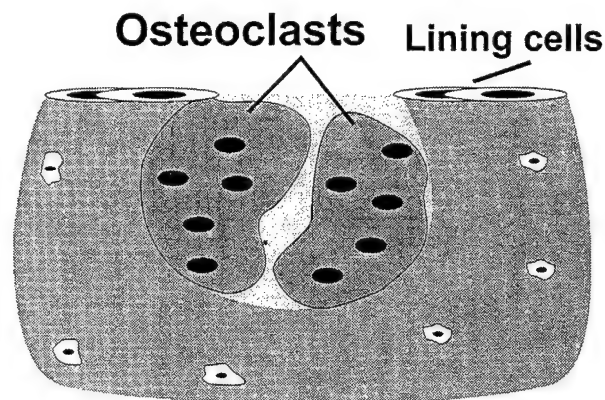
The overall rate of bone remodeling is determined by the number of remodeling units. During the bone remodeling sequence, bone formation follows bone resorption and as a consequence is coupled to bone resorption. Because of coupling, changes in the rate of bone resorption result in delayed corresponding increases or decreases in bone formation. Changes in bone mass (ΔM) for a given time interval are determined by the relationship $\Delta M = (\Delta R - \Delta F)_1 + (\Delta R - \Delta F)_2 + (\Delta R - \Delta F)_3 + \dots + (\Delta R - \Delta F)_N$ where N is the number of remodeling units, F is bone formation, R is resorption, and $R - F$ is the net difference between formation and resorption for a completed or partially completed remodeling unit. Thus, increasing the rate of bone remodeling will inevitably result in bone loss (because resorption precedes formation), which will be accentuated if bone formation does not completely refill the resorption cavity ($R - F < 0$).

Peak blood alcohol levels and duration of exposure to alcohol are two variables that are likely to influence the skeletal response to ethanol (Turner et al., 1998). However, there have been no experimental studies in human subjects and few in laboratory animals that have focused on dose-dependent changes in bone remodeling. Epidemiological studies suggest that infrequent to moderate alcohol consumption decreases the bone remodeling rate (N) without disturbing remodeling balance ($R - F$) (Fig. 1B), whereas

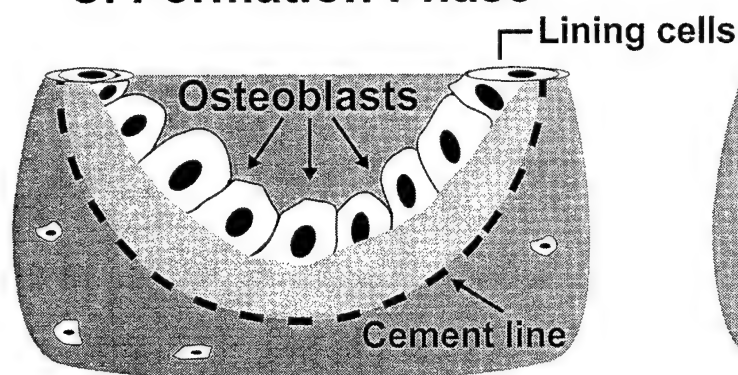
A. Quiescent Bone Surface



B. Resorption Phase



C. Formation Phase



D. Quiescent Phase

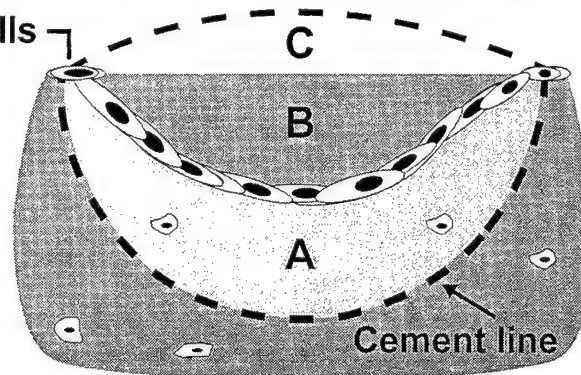


Fig. 1. The bone remodeling cycle. (A) Quiescent phase—inactive bone with surface lined with bone lining cells. Neither bone resorption nor formation is occurring on this region of bone surface. (B) Resorption phase—osteoclast-mediated bone resorption. The osteoclasts remove a discrete packet of bone, creating a lacunae. (C) Formation phase—osteoblasts form bone matrix that falls in the lacunae. The cement line defines the boundary between the newly formed bone and the surface of the lacunae excavated by the osteoclasts at the end of the resorption phase. (D) Quiescent phase—inactive bone surface that shows the completed remodeling cycle. The new surface may be underfilled (a), exactly filled (b), or overfilled (c), with the resorption lacunae reflecting a local decrease, no change, or an increase in bone mass, respectively. The most likely mechanism for alcohol-induced bone loss in adults is underfilling of the resorption lacunae during bone remodeling.

alcohol abuse decreases N and creates a negative remodeling balance (Fig. 1A). The exact dose response for either N or $R - F$ remains to be determined and is likely to be influenced by additional factors such as age, sex, and gonadal status.

The apparent contradictory effects of alcohol on bone mass in young men and postmenopausal women can be reconciled by considering the effects of drinking on bone remodeling. An imbalance in bone formation and resorption in young males in which the latter predominates would lead to gradual bone loss despite decreased bone remodeling. In contrast, an alcohol-induced decrease in bone remodeling in older women would slow bone loss relative to their peers despite the remodeling imbalance. In women, gonadal insufficiency after menopause greatly accelerates bone remodeling with a net increase in bone resorption (Heaney et al., 1978). Inhibitors of bone remodeling (such as estrogen) reduce the rate of bone loss (Felson et al., 1993). Thus, an inhibitory effect of moderate alcohol intake on initiation of the bone remodeling cycle would be con-

sistent with studies that report a relative (compared with age-matched women who are losing bone) improvement in bone mass in postmenopausal women.

Another type of bone turnover allows bone formation and bone resorption to be independently changed in magnitude and can lead to architectural changes. This process is called modeling, and it need not involve a coupled response between bone formation and resorption (Turner, 1994). The relative contributions of altered bone modeling and remodeling to the bone loss induced by chronic alcohol abuse are not known.

As eluded to, the relationship between bone resorption by osteoclasts and bone formation by osteoblasts is central to achieve and maintain a neutral bone balance. This is analogous to a biochemical pathway where positive (activator) and negative (inhibitor) factors play a critical role in regulating the balance between opposing processes in the system. Local signaling peptides (growth factors and cytokines) appear to act as the biochemical messengers between the two classes of cells (Wergedal et al., 1986). In

order for osteoclasts to resorb bone, first they have to be formed and then they have to become activated by contact with resorbable bone matrix (Vaes, 1988). The latter process requires removing both an osteoblast cell layer and an underlying layer of nonmineralized matrix (or osteoid tissue) (Vaes, 1988). It has been proposed that osteoblast lineage cells might regulate bone resorption in part by secreting enzymes to both remove the osteoid layer and activate osteoclasts (McSheehy and Chambers, 1986a; Vaes, 1988). The osteoclast, however, is responsible for resorbing the organic as well as mineral phase of bone.

There is evidence for communication from osteoclasts to osteoblasts to create tighter interaction between the two metabolic processes of formation and resorption (McSheehy and Chambers, 1986b; Vaes, 1988). The factors involved in this communication have been termed "coupling factors" (Linkhart et al., 1986). An osteoclast-derived factor, which affects osteoblasts, has not been identified. However, non-osteoclast-derived peptides have been implicated as coupling factors (Howard et al., 1981; Linkhart et al., 1986; Pfeilschifter et al., 1988). These peptides are produced by osteoblasts, trapped in bone matrix during bone formation, and quantitatively released by the resorbing activities of osteoclasts (Turner et al., 1988b). Such a mechanism is hypothesized to promote osteoblast activity to follow bone resorption. Recent studies have demonstrated that the final step in osteoclast differentiation is regulated by osteoblasts and certain marrow cells (Simonet et al., 1997). Alcohol may alter the rate of initiation of bone remodeling as well as the coupling between bone formation and bone resorption by disturbing the local expression of cytokines that mediate these processes. In support of this hypothesis, the expression of two important cytokines, insulin-like growth factor-I and tumor necrosis factor- α , have been shown to be altered in bone by alcohol (Turner et al., 1998).

LIMITATIONS OF HUMAN STUDIES

Human studies in alcoholics are often difficult to interpret. Most have been underpowered because of the relatively small number of patients studied and wide variations in age, duration and patterns of alcohol abuse, and other risk factors. The precise effects of alcohol on the human skeleton are not known because it is difficult to distinguish the specific effects of ethanol from comorbidity factors such as poor nutritional status, magnesium and zinc deficiency, reduced mechanical loading due to decreased exercise and weight loss, malabsorption related to chronic pancreatitis, cigarette smoking, and use of aluminum-containing antacids. It is difficult to get an accurate assessment of lifelong alcohol consumption, and inaccurate reporting can compromise interpretation of the results. The role of abnormal liver function is especially controversial, and investigators report bone loss (Bikle et al., 1985; Laitinen et al., 1992) as well as no bone loss (Crilly et al., 1988;

Harding et al., 1988) in alcoholics free of liver damage. One possible explanation for the variable results is that the age, weight, and nutritional status of the individual may be as important as the duration and magnitude of exposure to alcohol, with older (Bikle et al., 1993) and lighter weight (Crilly and Delaquerriere-Richardson, 1990) abusers being more subject to bone loss than younger and heavier individuals.

LABORATORY ANIMAL MODELS

Studies of the effects of ethanol on bone and mineral homeostasis suggest that laboratory animals can provide additional insight into the specific effects of alcohol. Alcohol given acutely results in transient hypocalcemia in rats and dogs (Peng et al., 1972; Peng and Gitelman, 1974). Alcohol given chronically to growing male rats decreases bone strength, bone density, and cancellous bone volume (Peng et al., 1988; Turner et al., 1988a, 1991; Wezeman et al., 1999). Alcohol also suppresses overall growth. When pair feeding was used to ensure comparable growth between controls and alcohol-treated rats, consumption of alcohol that comprised 36% of caloric intake for 3 weeks clearly inhibited bone formation at the tibial diaphysis, which delayed bone mineralization and decreased bone strength (Turner et al., 1987, 1991). Alcohol consumption did not alter serum concentrations of either 24,25(OH) $_2$ D or 1,25(OH) $_2$ D. Consumption of alcohol for 10 months resulted in cortical bone loss due to decreased bone formation (Turner et al., 1988a). Chronic alcohol consumption also resulted in cancellous osteopenia, but the cellular mechanism for the net bone loss was not established with certainty. After this long-term exposure to alcohol, serum 25(OH)D was increased but 1,25(OH) $_2$ D was decreased. These results demonstrate that the overall changes in bone and mineral homeostasis associated with long-term alcohol abuse in humans also occur in laboratory animal models.

Similarly, alcohol inhibits bone growth and reduces peak bone mass in female rats (Dyer et al., 1998; Hogan et al., 1997; Sampson 1998; Sampson and Spears, 1999; Sampson et al., 1996, 1997, 1998). Cessation of alcohol consumption did not result in sufficient catch-up growth to restore normal bone mass or mechanical properties (Sampson and Spears, 1999). As in growing males, alcohol induces osteopenia in female rats primarily by inhibiting bone formation (Dyer et al., 1998; Hogan et al., 1997; Sampson, 1998; Sampson and Spears, 1999; Sampson et al., 1996, 1997, 1998).

Alcohol did not inhibit bone formation or result in additional bone loss in growing ovariectomized rats (Kidder and Turner, 1998). Although one study reported a decrease in osteoclast number (Fanti et al., 1997), moderate to high consumption of alcohol did not prevent osteopenia induced by ovariectomy (Fanti et al., 1997; Kidder and Turner, 1998; Sampson and Shipley, 1997).

In most long-term studies, alcohol has been administered

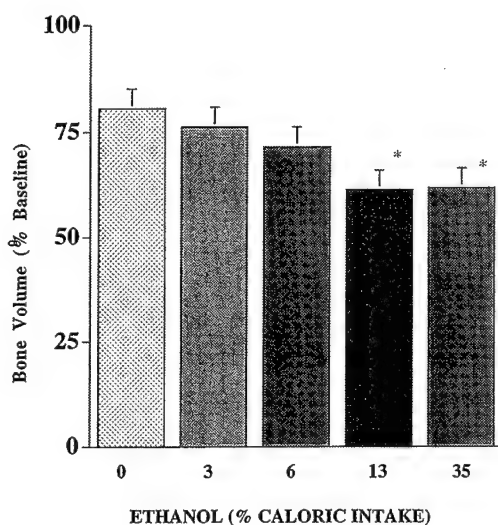


Fig. 2. Dose-response effects of alcohol on cancellous bone volume in female 8-month-old adult rats. A baseline group of animals was sacrificed at the start of the study. The treatment groups were fed a liquid diet that contained ethanol which comprised 0% to 35% of their caloric intake for 4 months. Cancellous bone volume in the treated groups was expressed as percentage of baseline control group, and the data were analyzed by ANOVA. There was dose-dependent decrease in cancellous bone volume at the proximal tibial metaphysis over and above the age-related bone loss. Data are mean \pm SE ($n = 8-11$). * $p < 0.05$ compared with age-matched rats fed the 0% ethanol control diet.

to rats in their diet. One study that sought to model binge drinking in rapidly growing rats by administering alcohol by gavage reported an increase in bone mass (Sampson et al., 1999). This unexpected finding suggests that additional factors such as age, gonadal status, or the pattern of consumption may be important in determining the overall skeletal effects of alcohol.

There are no published studies that report the long-term effects of alcohol on the skeleton of adult animals. However, a 4 month dose-response study in which ethanol was fed to female rats who were 8 months old at the start of treatment has been performed. Alcohol treatment resulted in a dose-dependent decrease in cancellous bone volume compared with baseline values or controls (Fig. 2). These findings demonstrate that chronic heavy alcohol consumption results in bone loss in the rat model. Especially disturbing was the finding that alcohol which comprised as little as 3% of the rats' caloric intake significantly reduced bone turnover (Turner et al., 2000).

CLINICAL SIGNIFICANCE OF MUSCULOSKELETAL DISORDERS

The clinical importance of musculoskeletal disorders in the general population is greatly underappreciated. Musculoskeletal complaints represent the number 1 and 2 reasons why patients see physicians and are hospitalized, respectively (Praemer et al., 1992). The annual direct and indirect costs of musculoskeletal disorders in the United States exceed \$125 billion. Direct treatment accounts for

less than half of the economic burden; morbidity, mortality, and the value of lost productivity account for the remaining costs.

There are more than 6 million bone fractures in the United States per year, of which roughly 5% do not heal properly and require additional and sometimes costly interventions. The consequences of fractures are especially severe in the elderly. Hip fractures are arguably the most devastating common fracture, with more than 275,000 occurring annually. Patients with hip fractures have a 20% 6 month mortality rate. Furthermore, an additional 20% will require long-term institutionalization (>1 year), and a similar percentage will face loss of mobility (i.e., dependence on wheelchair or walker) (Cummings et al., 1985).

The aging of the American population will lead to future increases in the incidence of osteoporotic fractures. Osteoporosis usually is caused by a chronic imbalance in the bone remodeling cycle where bone resorption is not adequately compensated for by subsequent bone formation. The onset of bone loss typically precedes the increased risk of fractures by one or two decades (Cummings et al., 1985; Praemer et al., 1992) and is asymptomatic during this interval. Insidious bone loss combined with the great difficulty of restoring bone to an osteopenic skeleton make it imperative to identify preventable risk factors for bone loss and to effectively intervene before clinical manifestations.

EFFECTS OF ALCOHOL ABUSE ON BONE MASS AND FRACTURE INCIDENCE

Until recently, the development of osteoporosis in men generally was perceived as an insignificant problem and often was not diagnosed, but male osteoporosis represents approximately one-third of the cases. Increasing longevity is likely to lead to future increases in the incidence of osteoporotic fractures in men as well as women. The development of osteoporosis in men often is associated with alcoholism. In one study, 45 of 96 young to middle-aged male alcoholics showed radiographic evidence of osteopenia (Spencer et al., 1986). Other studies have reported decreases in bone mass in male alcoholics by densitometry and histomorphometry of iliac crest bone biopsies (Bikle et al., 1985, 1993; Diez et al., 1994; Gonzalez-Calvin et al., 1993; Lalor et al., 1986; Pumarino et al., 1996; Schnitzler and Solomon, 1984). Histological studies suggest that alcohol abuse is associated with reductions in cancellous bone volume and thickness of individual trabeculae. However, not all studies have detected significant differences in bone mass between male alcoholics and nonalcoholics (Odvina et al., 1995; Pumarino et al., 1996).

Bone loss that results in atraumatic fractures in women is most commonly associated with gonadal hormone deficiency. There is little evidence for bone loss in female alcohol abusers (Laitinen et al., 1993). However, the great majority of studies performed in alcoholics to assess the effects of alcohol abuse on bone and mineral homeostasis

have been performed in men. As a result, this risk has not been established with certainty for women. Animal studies suggest that younger men and women who abuse alcohol share a similar risk but that postmenopausal women may be at a lower risk than elderly men.

Prospective, as well as retrospective, studies have been performed to establish an association between alcohol and bone mass. In contrast with studies in alcoholics, these population-based studies generally have shown no difference or a higher bone mineral density associated with alcohol consumption (Felson et al., 1995; Feskanich et al., 1999; Grainge et al., 1998; Hoidrup et al., 1999a,b; Holbrook and Barrett-Connor, 1993; Jouanny et al., 1995; Krogsgaard et al., 1995; Laitinen et al., 1992; May et al., 1995; Orwoll et al., 1996; Perry et al., 1999). This apparently beneficial effect of alcohol is most notable in women (Felson et al., 1995; Feskanich et al., 1999; Hoidrup et al., 1999a,b; Holbrook et al., 1993; Orwoll et al., 1996).

The effect of chronic alcohol abuse on fracture risk has not been studied extensively. Fractures were reported to be approximately four times as common in a series of 107 chronic alcoholics as in age-matched random controls (Kristensson et al., 1980). In another study, excessive alcohol consumption was identified in 7 men out of a series of 47 men sequentially referred to a metabolic bone center because of atraumatic fractures or radiographic osteopenia (Kelepouris et al., 1995). In a larger cross-sectional study, alcohol abuse also was identified as a significant risk factor for fractures in men (Kanis et al., 1999), and fractures were much more common in patients with alcoholic liver disease than those with various forms of nonalcoholic liver disease (Lindsell et al., 1982). In other studies, alcohol consumption was found to be not associated with fractures (Mussolino et al., 1998; Naves Diaz et al., 1997) or associated with decreased fracture risk (Nguyen et al., 1996), or the association was found to be to be sex dependent (Hoidrup et al., 1999a). In the study by Hoidrup et al. (1999a), alcohol abuse was associated with an increased fracture rate in men but not in women.

Numerous factors could contribute to the differences in fracture rate that have been reported, including the presence or absence of alcoholic liver disease and differences between the skeletal sites measured (e.g., ribs and vertebrae). One of the more important factors may be trauma (Johnson et al., 1984). Peris et al. (1995) found that most fractures in alcoholics were associated with trauma rather than osteopenia.

There have been few studies on the effects of alcohol on fracture repair. The limited available data suggest that alcoholism does not increase the incidence of nonunions, osteonecrosis, or other complications (Nyquist et al., 1997, 1998) but is associated with an increase in healing time for transverse fractures (Nyquist et al., 1997).

SUMMARY AND RECOMMENDATIONS

Alcohol abuse should be considered a risk factor for osteoporosis based on the frequent finding of a low bone mass, decreased bone formation, and increased fracture incidence in alcoholics. Alcohol also has been shown to reduce bone formation in healthy humans and animals and decrease proliferation of cultured osteoblastic cells. On the other hand, not all alcoholics exhibit a low bone mass. Furthermore, it has been difficult to demonstrate either alcohol-induced bone loss or increased fracture rate in population-based studies. Indeed, most have shown a positive association between alcohol and bone mass and no change or a decrease in fracture risk. Overall, the evidence generally supports a detrimental effect of chronic alcohol abuse on the skeleton of a subpopulation of men and a neutral or generally beneficial effect for moderate alcohol consumption, especially in women. This latter putative beneficial effect may be due to a reduction in the increase in bone remodeling that in part mediates age-related bone loss.

The following areas need additional research to resolve controversies, establish degree of risk, or develop countermeasures to prevent or reverse the detrimental skeletal effects of alcohol abuse.

Fracture Rate

Many alcoholics are osteopenic, and a low bone mass is an established risk factor for atraumatic fractures. Although there is no clear association between drinking and bone mass in the general population, the magnitude of risk can be determined only if the fracture incidence for alcoholics is known. Ideally, these data will distinguish between traumatic and atraumatic fractures to determine if osteopenia rather than an increased accident rate is the major contributing factor that leads to fracture in alcoholics (Blake et al., 1997; Nordqvist and Petersson, 1996).

Bone Turnover

Alcohol inhibits bone formation in humans and growing animals but there are important controversies regarding its effects on bone turnover. Is alcohol toxic to osteoblasts *in vivo*? Does alcohol antagonize osteoblast differentiation? Does alcohol act directly on the osteoblast to suppress bone matrix synthesis, and if there is direct inhibition, is it reversible? To what extent is the reduction in bone formation an indirect consequence of an overall reduction in bone remodeling? Does alcohol uncouple bone formation from bone resorption during bone remodeling? Do biochemical markers in blood and urine accurately reflect bone formation and bone resorption in alcoholics? Existing data in the literature both support and refute each of these possibilities. The answers to these questions are relevant to gaining insight into the etiology of alcohol-induced osteoporosis and are important to the rational development of counter-

measures. Future studies should not be limited to alcohol abuse nor should they be limited to one sex or one age. Studies that emphasize moderate drinking should be encouraged because of the huge potential impact on public health.

Mechanisms

The biochemical and molecular mechanisms of action of alcohol on bone cells are poorly understood. Animal and cell culture models are now available to advance our understanding of mechanisms, and such studies could lead to novel interventions and should be supported.

Countermeasures

Studies that investigate countermeasures to the detrimental effects of alcohol on bone and mineral metabolism are rare. Dramatic recent advances have been made toward prevention and reversal of postmenopausal osteoporosis by using pharmacological approaches. The possible application to alcoholics of approved drugs that inhibit bone resorption and drugs under development that stimulate bone formation should be investigated. Development of novel therapies specific to alcoholics also should be encouraged.

Animal Models

Good models for chronic alcohol abuse have been established. However, improved animal models for binge and underage drinking should be developed and validated. Although administration of high concentrations of alcohol to the diet of baby rats clearly has demonstrated that alcohol can antagonize bone growth, it is not at all certain that these findings are relevant to the important social and public health problems of underage drinkers. Specifically, there is an urgent need for models that better replicate the drinking patterns of adolescents, as well as moderate and binge-drinking adults. Animals could be used to investigate the respective effects of total alcohol consumed, peak blood levels, age of initiation, and frequency and duration of consumption on bone and mineral metabolism. Such studies are likely to provide important insight into the variability observed in alcoholics and moderate drinkers and should be encouraged.

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Moderate Alcohol Consumption Suppresses Bone Turnover in Adult Female Rats

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ABSTRACT

Chronic alcohol abuse is a major risk factor for osteoporosis but the effects of moderate drinking on bone metabolism are largely uninvestigated. Here, we studied the long-term dose-response (0, 3, 6, 13, and 35% caloric intake) effects of alcohol on cancellous bone in the proximal tibia of 8-month-old female rats. After 4 months of treatment, all alcohol-consuming groups of rats had decreased bone turnover. The inhibitory effects of alcohol on bone formation were dose dependent. A reduction in osteoclast number occurred at the lowest level of consumption but there were no further reductions with higher levels of consumption. An imbalance between bone formation and bone resorption at higher levels of consumption of alcohol resulted in trabecular thinning. Our observations in rats raise the concern that moderate consumption of alcoholic beverages in humans may reduce bone turnover and potentially have detrimental effects on the skeleton. (*J Bone Miner Res* 2001;16:589–594)

Key words: rat bone, alcohol abuse, bone formation, bone resorption

INTRODUCTION

ALCOHOLICS OFTEN have radiographic and histomorphometric evidence of osteopenia and a greatly reduced bone mineral density (BMD).^(1–3) Histomorphometric analysis of bone biopsy specimens and measurement of biochemical markers of bone metabolism have revealed consistent evidence that alcohol excess inhibits bone formation.^(4–13) The effects of ethanol on bone resorption are less certain; increases, decreases, and no change have been reported.^(2,4–7,11,13)

The inability to assign a role for bone resorption in mediating alcohol-induced bone loss highlights the difficulties associated with performing studies in alcoholics. Human studies often are difficult to interpret because of the small number of patients who can be studied and wide variations of the patient population in age, duration, and pattern of alcohol abuse and accompanying risk factors. It is difficult to distinguish the direct effects of ethanol from secondary factors such as magnesium and zinc deficiency,

reduced mechanical loading of the skeleton caused by decreased physical activity and weight loss, malabsorption caused by chronic pancreatitis, and skeletal abnormalities associated with increased cigarette smoking and increased use of aluminum-containing antacids. The role of abnormal liver function is especially controversial, with some investigators reporting bone loss in alcoholics free of liver disease and others reporting no bone loss.^(4,8,12,14–17) It is especially difficult to control for nutrition, in part because alcoholics have a larger caloric intake than their peers but frequently are underweight.^(18,19)

A rat model for alcohol abuse has been developed to circumvent the limitations of human studies. Weight and nutrition can be controlled carefully in this animal model. Growing rats who consume ethanol at a rate (adjusted for the difference in body mass) comparable with alcoholics develop osteopenia and other abnormalities in bone and mineral metabolism.^(20,21) All of the changes described in the rat model have been reported in alcoholic patients.^(2–4,6,7,11–13,15,17)

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The skeletal response to low and moderate alcohol consumption is relevant to a much larger segment of the adult population than is alcohol abuse but has not been studied extensively in either humans or laboratory animals. The present investigation was designed to investigate the long-term dose-response effects of ethanol on the skeleton of adult female rats. Specifically, the study was designed to determine the minimum consumption of ethanol required to induce bone loss.

MATERIALS AND METHODS

Animal experiment

Female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were received at 8 months of age (body weight [BW], 279 ± 3 g; mean \pm SE). Subsequent procedures performed on animals were approved by the Mayo Institutional Animal Care and Use Committee in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Rats were weight-matched into seven study groups ($n = 9-12$) comprised of four ethanol-treated groups (3, 6, 13, and 35% caloric intake), a control group fed ad libitum, a control group pair fed to the 6% ethanol-treated group, and a control group killed at the start of the experiment (baseline). As described for a previous study,⁽²²⁾ rats were housed individually in a temperature- and humidity-controlled animal facility on a 12-h light/dark cycle. During the first week of the study, all animals were acclimated to a modified Lieber-DeCarli liquid diet (Bio-Serve, Frenchtown, NJ, USA). This diet contains 1.3 g/liter calcium and 1.7 g/liter phosphorous. Protein, fat, and carbohydrates contribute 18, 35, and 47% of caloric intake, respectively. The alcohol-supplemented groups were subsequently fed, ad libitum, a liquid diet containing increasing concentrations of ethanol (95% vol/vol) until receiving the appropriate percent of total caloric intake at the end of the first treatment week. Treatment continued for 4 months. Rats were not given water. Control rats did not receive ethanol and were fed a liquid diet isocalorically supplemented with maltose/dextran, as per the manufacturer's instructions. All animal weights were recorded weekly for the last 12 weeks of the study. On the first day of ethanol treatment, all rats were injected with a 20 mg/kg BW dose of a bone fluorochrome (tetracycline-hydrochloride; Sigma, St. Louis, MO, USA) perivascularly at the base of the tail. The baseline control rats were killed 24 h later. The remaining rats were injected (20 mg/kg BW) with the fluorochromes calcein (Sigma) and demeclocycline (Sigma) 9 days and 2 days before death, respectively. Rats were anesthetized using ketamine and xylazine, weighed, and killed by decapitation. Tibiae were harvested, defleshed, and fixed by immersion in 70% ethanol for bone histomorphometry. Wet weights of uteri were recorded.

Cancellous bone histomorphometry

Tibiae were processed for plastic embedding without demineralization and sectioned as described.⁽²²⁾ Histomorphometry of cancellous bone was performed on unstained

longitudinal sections (5 μ m thick) in a standard sampling site in the proximal tibial metaphysis.⁽²²⁾ Terminology and abbreviations are consistent with the standardized histomorphometric nomenclature.⁽²³⁾

All measurements were conducted on an image-analysis system employing OsteoMeasure software (OsteoMetrics, Atlanta, GA, USA) as described.⁽²²⁾

The following measured and derived histomorphometric values were obtained: total cancellous bone area (BA) measured at a magnification of $\times 10$ in a metaphyseal sampling site divided by the total area of the tissue sampling site (2.88 mm²) and expressed as a percentage; cancellous BA was compared between the ad libitum and the pair-fed control groups to determine if there was a significant effect of diet on BA; and trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.S) were estimated using the method of Parfitt et al.⁽²⁴⁾

Fluorochrome-based measurements and derived values consisting of double-labeled perimeter, mineral apposition rate (MAR), and bone formation rate (BFR) were determined as described.⁽²²⁾ The measurements were performed at a magnification of $\times 10$.

The length of cancellous bone perimeter covered by osteoclasts was measured in toluidine blue-stained sections at $\times 20$, divided by total bone perimeter, and expressed as a percentage. Osteoclasts were morphologically distinguished as large, multinucleated cells with a foamy cytoplasm juxtaposed to bone surface.

Statistics

All values are expressed as means \pm SE. Significant differences between alcohol-treated groups and controls were determined by Fisher's protected least significant difference post hoc test for multiple group comparisons after detection of significance by one-way analysis of variance (ANOVA). Significance was considered at values of $p < 0.05$. Dose-response effects were evaluated by linear regression analysis.

RESULTS

Pair feeding had no effect on any measured value. For this reason, the pair-fed and ad libitum control groups were combined.

The effects of ethanol on BW; change in BW, food, and ethanol consumption; and uterine weight are shown in Table 1. The initial BWs did not differ between the treatment groups. Ethanol had minor effects on final BW and food consumption. The lowest concentration of ethanol (3% caloric intake) increased final BW and rate of change in BW as well as consumption of the diet, whereas the highest concentration (35% of caloric intake) significantly decreased food consumption and rate of change in BW. There was a nearly linear increase in total ethanol consumed per day as the concentration of ethanol was increased in the diet. Ethanol treatment tended to decrease uterine weight; the reductions were significant for the groups with 6% and 35% caloric intakes.

TABLE 1. BODY AND UTERINE WEIGHTS

Group	Initial BW (g)	Necropsy BW (g)	Change in BW (g/day)	Diet consumed (ml/day)	Ethanol consumed (ml/day)	Uterine weight (g)
Control	277 ± 5	321 ± 9	0.40 ± 0.06	67.8 ± 2.3	0	0.931 ± 0.047
3% Ethanol	279 ± 6	358 ± 15*	0.75 ± 0.10*	76.6 ± 1.5*	0.44 ± 0.01*	0.946 ± 0.069
6% Ethanol	281 ± 5	339 ± 12	0.54 ± 0.12	69.3 ± 1.4	0.80 ± 0.02*	0.778 ± 0.063*
13% Ethanol	272 ± 9	323 ± 15	0.47 ± 0.08	64.1 ± 1.3	1.60 ± 0.03*	0.835 ± 0.078
35% Ethanol	285 ± 6	291 ± 7	0.06 ± 0.03*	61.5 ± 2.4*	4.12 ± 0.16*	0.722 ± 0.045*

Values are mean ± SEM.

* $p < 0.05$ treatment ($n = 9-12$) versus control ($n = 20-22$).

TABLE 2. CANCELLOUS BONE ARCHITECTURE

Group	BA/TA (%)	Tb.Th (μm)	Tb.N (mm^{-1})	Tb.S (μm)
Baseline	25.6 ± 1.3	70.1 ± 1.9	3.7 ± 0.2	208 ± 13
Control	20.6 ± 1.2	64.9 ± 2.6	3.2 ± 0.1	260 ± 15
3% Ethanol	19.5 ± 1.4	62.5 ± 3.0	3.1 ± 0.1	267 ± 16
6% Ethanol	18.3 ± 1.7	57.6 ± 2.3*	3.2 ± 0.2	274 ± 26
13% Ethanol	15.7 ± 2.3*	52.5 ± 2.4*	2.9 ± 0.4	359 ± 73
35% Ethanol	15.8 ± 1.0*	50.9 ± 1.7*	3.1 ± 0.1	281 ± 20

Values are mean ± SEM.

* $p < 0.05$ treatment ($n = 8-11$) versus control ($n = 19$).

The effects of ethanol on static bone histomorphometry are summarized in Table 2. BA/tissue area (TA) was significantly decreased in the 13% and 35% caloric intake groups and Tb.Th was decreased for intake levels at and above 6%. Tb.N and Tb.S were not significantly influenced by ethanol.

The effects of ethanol on cancellous bone dynamic and cellular histomorphometry are summarized in Table 3. Ethanol treatment decreased mineralizing perimeter (M.Pm) and BFR (perimeter referent). Ethanol had no effect on MAR. All concentrations of ethanol treatment reduced osteoclast perimeter (Oc.Pm) to a similar magnitude.

We were unable to measure longitudinal bone growth because of inadequate separation between the tetracycline label given at the start of the experiment and the mineralized hypertrophic cartilage.

The dose-response effects of alcohol are summarized in Table 4. Linear regression revealed significant dose-dependent decreases in BA/TA, Tb.Th, M.Pm, and BFR. There was no dose-dependent effect of alcohol on BW, uterine wet weight, Tb.N, Tb.S, MAR, or Oc.Pm.

DISCUSSION

The observed results are disturbing because we did not observe a no-effect dose for alcohol consumption. Pronounced changes in bone metabolism were observed at the lowest consumption level of alcohol. Dietary intake of alcohol comprising as little as 3% of total calories dramatically reduced histological indices of bone turnover. Higher

consumption levels of alcohol resulted in alterations in trabecular architecture and even net cancellous bone loss.

Analysis of the cancellous bone architecture revealed that alcohol-induced bone loss was caused by a reduction in Tb.Th; Tb.N was not altered. Oc.Pm was not increased, indicating that cancellous bone loss was caused by a disturbance in the bone remodeling balance rather than increased bone remodeling. Indeed, the present results indicate that alcohol consumption results in reduced bone remodeling as evidenced by the decreases in histomorphometric indices of bone resorption (Oc.N) and bone formation (M.Pm).

Cancellous bone remodeling occurs when focal resorption (remodeling unit) is initiated on a previously quiescent trabecular surface.⁽²⁵⁾ A small amount of bone is resorbed and the resulting resorption lacuna is filled shortly thereafter as a result of new bone formation. There are two cellular mechanisms that could lead to trabecular thinning: (1) excessive erosion during the resorption phase and (2) incomplete refilling of the erosion cavity during the formation phase. The maximum inhibition of osteoclast number was achieved at the lowest level of alcohol consumption, whereas M.Pm showed a striking dose-dependent decrease. These findings suggest that incomplete filling of the erosion cavity during the formation phase of the remodeling cycle is the more likely cellular mechanism for trabecular thinning. The MAR was not altered, suggesting that alcohol inhibits onset of the bone formation phase of the remodeling cycle but not its continuation once initiated. These results are consistent with Dyer et al.⁽²⁶⁾ who concluded that alcohol

TABLE 3. FLUOROCHROME AND OSTEOCLAST MEASUREMENTS

Group	M.Pm/B.Pm (%)	MAR ($\mu\text{m}/\text{d}$)	BFR/BV (%/d)	Oc.Pm (%)
Control	11.05 \pm 0.75	0.94 \pm 0.04	0.325 \pm 0.019	15.77 \pm 1.15
3% Ethanol	5.79 \pm 1.07*	0.92 \pm 0.02	0.169 \pm 0.032*	10.78 \pm 1.85*
6% Ethanol	5.14 \pm 0.78*	0.91 \pm 0.03	0.171 \pm 0.031*	10.62 \pm 1.18*
13% Ethanol	3.71 \pm 0.77*	1.01 \pm 0.04	0.150 \pm 0.035*	10.66 \pm 0.99*
35% Ethanol	2.37 \pm 0.56*	0.86 \pm 0.04	0.077 \pm 0.019*	10.09 \pm 1.52*

Values are mean \pm SEM.

* $p < 0.05$ treatment ($n = 11$) versus control ($n = 18-20$).

B.Pm, bone perimeter.

TABLE 4. DOSE-RESPONSE EFFECTS OF ETHANOL ANALYZED BY LINEAR REGRESSION

Measurement	r Value	p Value
Necropsy BW (g)	—	NS
Uterine wet weight (g)	—	NS
BA/TA (%)	0.31	0.0001
Tb.Th (μm)	0.43	0.0012
Tb.N (mm^{-1})	—	NS
Tb.S (μm)	—	NS
M.Pm/B.Pm	0.77	0.0001
MAR ($\mu\text{m}/\text{d}$)	—	NS
BFR (%/d)	0.79	0.0001
Oc.Pm	—	NS

B.Pm, bone perimeter.

inhibits osteoblast proliferation and activity in the rat. This observation also is consistent with in vitro studies showing that ethanol delays recruitment of osteoblasts but has little effect on bone matrix protein gene expression and peptide secretion by mature osteoblasts.^(27,28) Similarly, ethanol did not increase apoptosis in vitro,⁽²⁵⁾ suggesting that osteoblast lifespan is unaffected.

At first glance, the observed nondose-dependent reduction in uterine weight in rats fed some doses of ethanol suggests that gonadal insufficiency contributes to the bone changes. However, the histological changes are not consistent with this possibility. Ovariectomy results in greatly elevated bone turnover^(29,30) whereas alcohol reduced bone turnover. In addition, the pattern of bone loss differs from ovariectomy. Gonadal insufficiency decreases Tb.N, whereas ethanol resulted in a decrease in Tb.Th.⁽²²⁾ The mechanism for the uterine atrophy is not clear but alcohol has been reported to induce a variety of pathophysiological changes in the uterus, including uterine atrophy.⁽³¹⁾ Furthermore, we have identified numerous genes in which expression is altered dramatically after acute administration of 1 mg/kg of ethanol, suggesting that alcohol has direct effects on the uterus (Turner et al., unpublished results, 2000).

Studies performed in growing male^(21,32-35) and female^(36,37) rats indicated that chronic consumption of large doses of alcohol in the diet suppresses bone growth. The resulting relative osteopenia is caused by the failure to acquire a normal bone mass and is relevant to juvenile

alcohol abusers. The present study in older rats is more relevant to adult humans and shows alcohol-induced bone loss in rats. Our failure to detect measurable longitudinal bone growth at the proximal tibial growth plate provides definitive evidence that the observed changes were not influenced by growth. The present study also differs from previous work in that it attempts to model moderate as well as abusive alcohol consumption.

There is no universal agreement as to what constitutes moderate drinking. Additionally, the level of ethanol consumption in rats in this study cannot be related directly to humans because the rates of metabolism differ between the two species. The variables that must be considered and evaluated in these studies include: total ethanol consumed, the percent caloric intake contributed by ethanol, and peak blood levels of ethanol.

The low-dose group (3% caloric intake) consumed approximately 0.4 ml ethanol/day. On a body mass basis, this would be equivalent to approximately three standard drinks by a 50-kg woman, which is on the high end of moderate alcohol consumption. However, relative to caloric intake, this level of consumption would be the equivalent of <0.5 daily drinks, which is on the low end. Blood ethanol levels may be more important than the absolute amount of alcohol consumed. Ethanol administered at 35% of caloric intake resulted in measured blood ethanol levels of 0.09–0.15%.^(21,32,33,36) The measured levels are likely to underestimate peak blood alcohol levels because sequential measurements have not been performed throughout the rat feeding cycle. Nevertheless, these levels are near to or exceed the impairment level, which generally is considered to be between 0.08 and 0.10%. This high blood alcohol level contrasts with the 3% caloric intake dose rate, which results in blood alcohol levels below the assay detection limit. Taken as a whole, the data suggest that our dose range in the rat extends from the human equivalent of low-moderate to alcohol abuse.

Bone formation generally is reduced in alcoholics.^(3,4,7,17) Additionally, administration of ethanol to healthy volunteers results in an acute decrease in serum osteocalcin levels.⁽⁸⁻¹⁰⁾ The similarity between our results and those seen in humans indicates that in addition to being a good model for alcohol abuse in adults, the mature rat also may be predictive for the skeletal effects of moderate drinking.

The implications of a reduction in bone turnover in moderate drinkers may depend on age and other factors. On one

hand, ethanol consumption by adolescents might reduce peak bone mass, which would predispose the individual to osteoporosis. On the other hand, a reduction in bone turnover is likely to reduce the risk of osteoporosis in postmenopausal women because these individuals are losing bone at a rapid rate because of, in part, elevated bone turnover. This speculation is supported by epidemiological data indicating that postmenopausal moderate drinkers have a higher bone mass than abstainers.⁽³⁸⁻⁴¹⁾ It also is supported by studies in ovariectomized rats, which show that ethanol does not accelerate the bone loss associated with gonadal insufficiency and may reduce osteoclast number.^(22,42,43)

Neither moderate nor high doses of alcohol prevented bone loss in ovariectomized rats.^(22,42,43) Ovariectomy is likely to result in a more extreme depletion of gonadal hormones than menopause. Epidemiological studies suggest that estrogen replacement accentuates the putative beneficial skeletal response to alcohol in postmenopausal women.⁽⁴¹⁾ Thus, it is possible that the combined antiremodeling actions of estrogen replacement and alcohol have additive effects in women and rats.

In summary, these studies in rats show that alcohol consumption results in dose-dependent bone loss and decreased bone turnover. A no-effect dose for alcohol was not observed. The findings in rats suggest that even moderate levels of alcoholic beverage consumption in humans may reduce bone turnover and potentially have detrimental effects on the skeleton.

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Animal Models For Osteoporosis

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Introduction

Laboratory animals have played a major role in the unprecedented recent improvements in the management of osteoporosis. They have contributed to enhanced knowledge of the etiology of osteoporosis. Additionally, animals have been essential for preclinical evaluation of the efficacy and safety of interventions intended to prevent and/or reverse bone fragility. The purpose of this review is to evaluate the strengths and weaknesses of the most well-established animal models for osteoporosis as well as the methods used to measure bone quantity, quality and turnover in animals. For a more comprehensive discussion of animal models, see Kimmel [1] and Geddes [2].

Goals of Animal Models for Osteoporosis

The ideal laboratory animal model would replicate a human condition with an absolute degree of fidelity. Unfortunately, this goal has not been achieved for osteoporosis, in part because fracture risk has not been reproduced in animals, and it has proven difficult to ascertain the true degree of correspondence between the mechanisms which lead to the bone changes in the animal model and its human counterpart.

Bone mass, although relatively easy to measure, is an insufficient endpoint for judging the fidelity of an osteoporosis model because fracture is the clinical outcome of osteoporosis and it is very important to determine the cause of the bone fragility leading to fracture. Osteopenia can be localized or general, involve cortical and/or cancellous bone, and result from an inhibition of bone growth as well as a net increase in bone resorption. An animal model which develops relative osteopenia because of a disturbance in bone growth is not valid as a model for adult onset bone loss.

Similarly, species differences at the cellular and biochemical levels may negatively influence the usefulness of an animal model.

An impressive number of factors that influence bone metabolism have been reported (Fig. 1) and certainly many others remain to be discovered. Consideration of the astronomical number of possible combinations of these factors leads to the conclusion that a similar phenotype could result from more than one distinct factor or combination. How then can we ever know the degree of correspondence of the mechanism(s) which mediate osteopenia in humans and laboratory animals?

The extent to which the underlying signaling pathways which regulate bone mass are conserved between species can rarely be known with certainty. It is, however, possible to objectively evaluate the usefulness of an animal model by evaluating the extent to which similar events, such as hormonal deficiency or aging, lead to similar metabolic, cellular and architectural changes in humans and the animal model. This approach is usually straight forward when applied to a single change (e.g., hormonal deficiency) but becomes much more difficult when trying to model complex processes such as aging. The ultimate test of an animal model's utility is its ability to successfully predict an outcome in people. A final consideration when evaluating animal models is practicality. High cost and limited availability will prevent the widespread adoption of an otherwise promising model.

The Use of Laboratory Animals as Models for Osteoporosis

Osteoporosis is a condition which is characterized by skeletal fragility caused by reduced bone mass and

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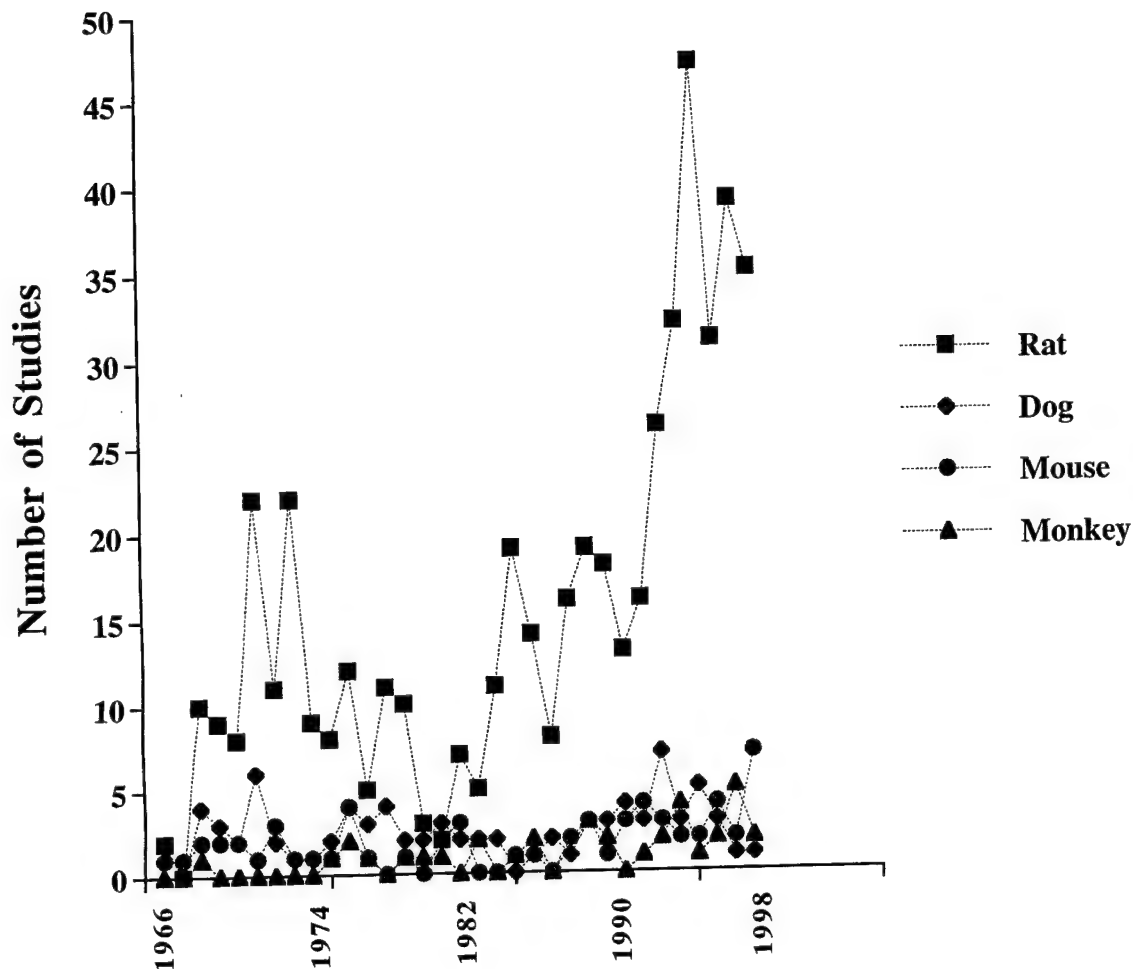


Fig. 2. A compilation of publications using animals as models for osteoporosis between 1966 and 1998. The number of publications is shown by year for rats, mice, dogs and monkeys.

The Growing Rat Model

A low peak bone mass is considered to be an important risk factor for development of osteoporotic fractures later

in life. The growing rat is a useful model for evaluating the effects of endocrine, nutritional and other environmental factors on peak bone mass. The young rapidly growing rat is appropriate for studies designed to

Table 1. Animal models for investigation of the etiology, prevention and treatment of osteoporosis

Model	Recommended	Conditionally recommended
Establishment of peak bone mass	Mouse, rat	
Genetic contribution to osteoporosis	Mouse	
Sexual dimorphism of skeleton	Rat	Mouse ¹
Postmenopausal osteoporosis	Rat	Dog, ² mouse, ³ primate ⁴
Fracture repair	Dog, rat	
Disuse	Dog, rat	Mouse ²
Steroid-induced bone loss		Mouse, ² rat ^{2,3}
Alcohol abuse-induced osteoporosis	Rat	
Senile-related bone loss		Mouse, ^{1,2} rat, ^{2,3} primate ⁴

¹Strain dependent.

²Insufficient characterization and/or controversial.

³Important differences compared to human response.

⁴Impractical for most investigators.

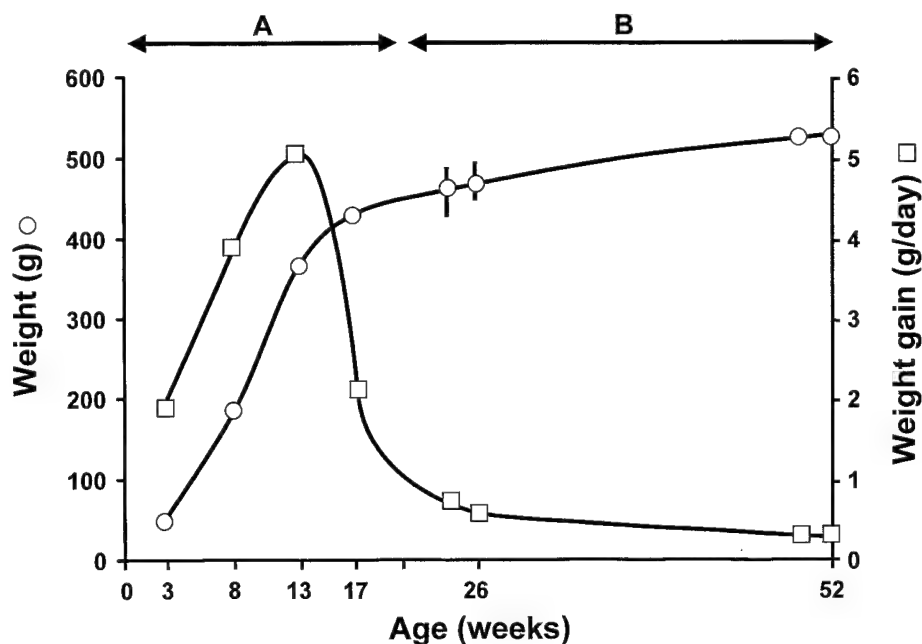


Fig. 3. A representative curve for the male Sprague Dawley rat. Region A corresponds to the rapid growth interval. Region B corresponds to the slow growth interval. Rats gradually increase in weight following cessation of skeletal growth (not shown) which occurs in the male at about 12 months of age and somewhat earlier in the female. Absence of error bars represent error is less than thickness of the line.

investigate factors related to peak bone mass but is a poor model for the adult human skeleton because skeletal growth is mediated by cellular processes which are not active in adults.

A growth curve for male Sprague Dawley rats is shown in Fig. 3. As in humans, female rats grow more slowly than males and obtain a smaller peak bone mass [3]. The rapid growth phase of the growth curve (Fig. 3, region A) occurs during the first 6 months of life. Not only are the rats growing rapidly during this interval but the growth rate changes continuously. The rate of weight gain reaches a peak shortly after puberty (6 weeks of age) and then declines rapidly with increasing age. The rapid growth phase is followed by much slower weight gain (Fig. 3, region B).

The Skeletally Mature Rat Model

The authors have frequently witnessed the unsubstantiated criticism of the rat as a model for the adult human skeleton, at scientific meetings and in manuscript and grant reviews, because of alleged continuous bone elongation and lack of bone remodeling in rats. Despite the frequency of these criticisms, we have been unable to identify any experimental studies reporting either lifelong growth of rat bone or an inability of this species to remodel bone. Indeed, there is compelling evidence for

basic multicellular unit-(BMU) based endocortical and cancellous bone remodeling in rats [4]. There is also conventional X-ray imaging-based evidence for epiphyseal closure in rats; the precise timing of which was found to be bone- and growth plate-dependent [5]. Using fluorochrome labeling, a more sensitive method, the authors have not been able to consistently detect longitudinal bone growth in hindlimb long bones of female rats over 8 months old or in male rats over 12 months old. Bone elongation therefore appears to be largely confined to the initial third of a rat's expected lifespan, a relative growth period which is longer than in humans, but not markedly so. High resolution micro-computed tomography (μ CT) of aged female rats has verified the fluorochrome-based results by clearly demonstrating bone bridging between the metaphysis and epiphysis [6], rendering any small amount of residual growth plate cartilage incapable of mediating longitudinal bone growth. These observations support use of the rat as a model for the adult human skeleton provided that either animals with fused growth plates are used, or it is demonstrated experimentally that growth does not influence interpretation of the data.

Rat Models for Osteoporosis

The observation that acute ovarian hormone deficiency leads to elevated cancellous bone turnover dramatically

increased interest in the rat as a model for postmenopausal osteoporosis [7]. Subsequent studies showing that ovariectomy results in cancellous and cortical bone loss have led to the widescale adoption of this model [3]. Similar bone loss can also be induced in female rats by LHRH agonists and estrogen receptor antagonists [8,9]. These alternatives to ovariectomy are reversible and have proven to be very useful for investigation of the bone loss associated with endocrine treatment of endometriosis. The predictive value of the ovariectomized rat is illustrated by the initial recognition of tissue selective actions of tamoxifen and other estrogen receptor ligands in that model. Subsequent confirmation in humans and development of selective estrogen receptor modulators (SERMs) for prevention of osteoporosis were a direct result of the initial animal observations [10,11].

The cancellous osteopenia which occurs following ovariectomy in rapidly growing rats is primarily due to altered bone growth and thus is mediated by a mechanism which differs significantly from postmenopausal bone loss [12]. In contrast, ovariectomy of skeletally mature rats is similar to menopause in that the surgery leads to cancellous and endocortical bone loss which is due primarily to abnormal bone remodeling.

The rat has been extensively used as a model for disuse osteoporosis. Disuse has been induced by unilateral sciatic nerve section, tendonotomy, unilateral limb casting, hindlimb unloading and spaceflight [13–17]. Each of these seemingly dissimilar methods results in similar skeletal changes, implying that the effects on bone are primarily due to unloading. These models have been used to study the etiology of disuse osteoporosis in growing and mature rats as well as to evaluate the efficacy of potential interventions.

Alcohol abuse is one of the most important “life style” risk factors for osteoporosis. Histological changes in the skeleton of alcohol-dependent rats [18] were later identified in alcoholics providing evidence that the species is useful for predicting human outcome. The major use of this model has been to better understand the etiology and severity of alcohol-induced bone loss [19].

Generalized age-related bone loss begins in men and women during their fifth decade, continues unabated through the remainder of life, and ultimately is responsible for senile osteoporosis. There is no compelling evidence that similar bone loss occurs in rats. However, localized bone loss may occur in the rat and aged gonadectomized rats develop severe osteopenia [6].

The usefulness of the rat as a model for glucocorticoid-induced osteoporosis is unclear. It is well established that glucocorticoids inhibit overall growth and bone formation in the rat. As a result, young rats may

develop osteopenia relative to normal growing controls. However, this relative osteopenia does not accurately model glucocorticoid-induced bone loss in adult humans. Some rat studies have shown an inhibition of bone remodeling by glucocorticoids, leading to a local increase in bone mass [20,21]. The transient increase in bone resorption and rapid severe bone loss which characterizes the pathogenesis of glucocorticoid-induced osteoporosis in humans are generally not apparent in rats. There have been associations between glucocorticoids, increased bone resorption, and reduced bone density [22,23], but definitive reports of bone loss are lacking. Interpretation of these results may have been complicated by the inhibitory effects of high doses of glucocorticoids on reproductive hormones [24]. The many discrepancies in the published literature suggest that the effects of glucocorticoids on bone turnover in the rat are inadequately understood to confidently recommend this species as a model for steroid-induced osteoporosis. Unfortunately, no other animals are clearly better.

Mouse

The mouse has similar growth characteristics to the rat. At 1/10 the mass of the rat, the advantages and disadvantages of its small size are even more pronounced.

The mouse is the premier laboratory animal model for studying the genetic contribution to peak bone mass and age-related bone loss [25]. There are numerous well characterized mouse strains with differences in bone mass and response to co-morbidity factors. Additionally, transgenic technology allows the purposeful manipulation of targeted gene expression [26]. There is a long and growing list of transgenic mice with perturbed bone metabolism (Table 2). Improvements in the ability to dynamically regulate genes in specific cell types will further increase the power of the mouse model. These genetic manipulations are not without pitfalls when applied to osteoporosis. Demonstration that a gene is associated with bone mass in the mouse does not necessarily mean that it has any role in the pathogenesis of osteoporosis. It will be essential to demonstrate that there is a cause and effect relationship in humans.

Ovariectomy results in cancellous osteopenia and accelerated bone turnover in the mouse. However, there are also clear differences between human and mouse physiology regarding the actions of estrogens and estrogen analogs such as tamoxifen [27]. While these species differences do not necessarily contraindicate its use, the ovariectomized mouse model should be approached with extreme caution.

The mouse is a promising model for age, disuse, and possibly even glucocorticoid-induced osteoporosis [28–

Table 2. Some transgenic mouse models exhibiting perturbed bone metabolism

Knock-out	Skeletal phenotype
Activating transcription Factor-2 (ATF-2)	Dwarfism, chondrodysplasia
Alkaline phosphatase	Decreased NOc; resembling infantile hypophosphatemia (rickets, osteomalacia)
c-abl	Reduced NOb and AOb; similar to Type II osteoporosis
Bcl-2	Dwarfism; pseudo-woven bone formation
Biglycan	Reduced BMD, thinner cortex—osteopenia
Bone sialoprotein	Reduced ossification, abnormally large incisors, smaller suture and bone marrow spaces in calvaria
Cathepsin K	Osteopetrosis
Ca sensing receptor	Stunted skeletal growth, reduced cortex; multiple fractures-osteopenia
Cbfa1/PcBp2 α A	— / — Fatal; open fontanelles & sutures; blocked intramembranous and endochondral ossification
CSF 1 (macrophage)	Osteopetrosis
Dopamine transport gene	Smaller cross-sections and lengths of long bones, diminished mechanical strength
Estrogen receptor alpha	Bone loss with OVX; no phenotype in SHAM mouse; reduced femur lengths in males
Estrogen receptor beta	Increased cortical cross-sectional area and BMC of femur; unchanged cancellous bone density
c-fos	Osteopetrosis
FGF receptor 3	Kyphosis & scoliosis; accelerated and prolonged bone growth
24-hydroxylase	In 2nd generation homozygotes osteomalacia at sites of intramembranous ossification
Interleukin 1 receptor	Protection against OVX-induced bone loss
Link protein (Crtl 1)	Dwarfism; craniofacial abnormalities
Matrix Gla protein	Extensive calcification
Matrix metalloproteinase 14	Dwarfism; osteopenia
5-lipoxygenase	Thicker cortex: partial protection against OVX-induced bone loss; stiffer but more brittle bones
Npt2, renal specific	Resembling Hypophosphatemic rickets
NF κ β	Osteopetrosis
oim (— / +)	Reduced mechanical strength-mild (type 1) osteogenic imperfecta.
Osteocalcin	Defective bone maturation
Osteoprotegerin	Osteoporosis
RANKL	Osteopetrosis
Procollagen type II alpha 1 (Col2 α 1)	Absence of endochondral ossification
Prolactin receptor	Reduced mineral apposition rate
PTH-PTHrP and PTH-PTHrP receptors	Osteochondrodysplasia
Pu.1	Osteopetrosis
Src	Osteopetrosis
Tartrate-resistant acid phosphatase	Osteopetrosis
Thrombospondin 2	Increased cortex
TNF receptor associated Factor 6 (TRAF6)	Osteopetrosis
TGF β	Reduced BMD, trabecular connectivity
Vitamin D receptor	Impaired bone formation; similarities to Type II Rickets
Over-expression	Skeletal phenotype
BMP receptor	Retarded ossification
Brain natriuretic peptide	Enhanced endochondral ossification, elongated growth plate
Calcitonin gene-related peptide	Increased bone density
Cyclin D1	Chronic to mild to moderate hyperparathyroidism
FGF receptor 3	Dwarfism
c-fos-jun (double)	Higher frequency of osteosarcomas
c-fos	Osteosarcomas, chondroblastic
HTLV-1	Increased bone turnover resembling Paget's disease
Human growth hormone	Increased BMD, cortex and mechanical strength
β 1 Integrin	Excessive bone resorption and increased NOc on endocranial surfaces
IL-4	Cortical and cancellous osteopenia
IL-6	Hypercellularity, focal cell proliferation, distorted bone formation in calvaria
Measles virus receptor	Increased NOc
PTHrP-collagen II	Jansen's metaphyscal chondrodysplasia, delayed endochondral ossification
TGF β 2	Increased bone turnover leading to bone loss
TRAF6	Osteopetrosis
Truncated TGF β 2 receptor	Increased cortex, cross-sectional area, trabeculae
Thymidine kinase	Osteoblast ablation-osteopenia
TNF receptor fusion protein	Protection from OVX-induced bone loss
Vitamin D receptor	Increased cross-sectional area, mechanical strength

30]. However, these models have been inadequately characterized to recommend them without qualifications.

Dog

Rodents are generally not suitable as models for intracortical bone remodeling. Larger animals such as the dog are more appropriate for these studies because they have well developed Haversian remodeling. The large animal also has major advantages as a model for highly localized bone fragility such as that associated with stress shielding by orthopedic implants [31]. The dog is also a well established laboratory animal model for more generalized disuse. In contrast, the dog is not widely used as a model for postmenopausal osteoporosis. Whereas some investigators have detected bone loss following ovariectomy, with or without concurrent hysterectomy, other investigators have detected no changes [1-3]. The relative insensitivity and inconsistent response of the dog skeleton to decreased gonadal hormones may be due to the six-month interval between periods of luteal activity. The large size and relatively long life span also discourage the use of the dog model because of the increased cost of maintaining the animals as well as administration of larger quantities of expensive and/or dangerous chemicals. An additional consideration is the reduced availability of molecular probes specific to dogs compared to rats and mice.

Primates

Several species of monkeys and apes have been used as models for osteoporosis. Monkeys and apes are generally more similar to human physiology than the more commonly used animal models for osteoporosis. The most compelling evidence for age-related osteopenia in an animal model is in monkeys [32]. Unfortunately, bone loss was not observed in monkeys less than 30 years old, severely limiting the practical application of what otherwise would be an excellent model for aging. Monkeys are an established model for disuse osteopenia but they do not offer many significant advantages over the dog [33]. One advantage that monkeys and apes have over other large animals is the availability of molecular probes. Because of the species similarity, many human probes are suitable for use in monkeys. Ovariectomy results in bone loss in monkeys raised in captivity [34]. However, recent studies in monkeys reared primarily in the wild failed to demonstrate bone loss following ovariectomy. The use of monkeys and apes as a model for osteoporosis is greatly limited by their expense, long life span, limited availability, and ethical concerns.

Evaluation of the Osteopenic Skeleton of Animal Models

The choice of methods to evaluate bone mass, architecture and metabolism are as important as the choice of the animal model. Endpoints which are routinely studied in human subjects are useful for evaluating the fidelity of the animal model. The principal purpose of the animal model, however, is to extend knowledge beyond that which can be obtained in humans by employing more sophisticated and/or invasive methods than are generally available to human studies.

Densitometry. Single- and dual-photon densitometry are commonly used in laboratory animals [15,35] to measure bone mineral density (BMD) and bone mineral content (BMC). The resolution has improved steadily during the last decade to where it is now possible to measure individual bone compartments in animals as small as a mouse. BMD measurements reported in animals are generally assumed to be comparable to those routinely obtained in adult human subjects and changes are usually interpreted as reflecting changes in bone mass. This interpretation is often not valid. BMD has no fundamental physical meaning; BMD is the mineral content of the bone normalized to an apparent cross-sectional area. Interpretation of BMD changes in growing animals is especially difficult because cross-sectional area and mineral content are both changing over time and are often differentially influenced by treatment (e.g., ovariectomy). As a result, BMD and bone mass can change in opposite directions.

In contrast, densitometry provides quantitative measurement of material (BMC) which is directly related to bone mass. Because of the inherent difficulties of interpreting BMD, BMC is the more informative endpoint and should always be reported.

The recent application of peripheral computerized tomography (pQCT) and high-resolution μ CT to assess bone changes in living animals provides the investigator with powerful new imaging techniques [36]. These instruments are capable of significantly higher resolution than currently used densitometric methods. The 3-dimensional architecture of bone can be studied over real time in small animals at a resolution capable of visualizing individual trabeculae. However, reconstructing images using voxal (3-dimensional equivalent of a pixel) dimensions (which are similar in length to trabecular thickness) causes errors, due to partial volume effects, greatly limiting the amount of architectural information that can be derived from these reconstructions. Radiation exposure, which increases as a cubic function compared to a linear increase in resolution, is

the principal factor limiting further improvements in resolution in living animals.

Much higher resolution can be obtained when μ CT is applied to tissues *ex vivo* because high radiation exposure is no longer a concern. Detailed 3-dimensional architectural structure can be obtained from the reconstructions as well as bone density. Mechanically loaded bone regions as small as individual trabeculae can be compared to the unloaded bone and the mechanical properties can be calculated after measuring the deformation, providing an alternative method to finite element modeling. Recent studies indicate that μ CT can detect local changes in bone density where bone-seeking heavy metals have been deposited following acute administration. This finding suggests that in the future μ CT can be used to measure bone formation in 3-dimensions using principles analogous to fluorochrome-based light microscope 2-dimensional measurements [36].

Biochemical markers. Analysis of mineral homeostasis can be performed in laboratory animals more easily than in humans, using a variety of *in vivo* and *ex vivo* approaches. The mineral (Ca, P, Mg) content of blood and urine are easily measured and radioisotopes can be administered as tracers. Additionally, *ex vivo* studies can be used to extend the capabilities of human studies to evaluate transport of minerals across the intestinal mucosa.

In contrast, the availability of biochemical markers of bone metabolism is generally more limited for animal models than humans. As a consequence, human assays have frequently been adapted to animals with loss of specificity and sensitivity. Markers for osteoblast differentiation and activity (alkaline phosphatase and osteocalcin) and collagen breakdown products are the most common biomarkers of bone metabolism. These markers are useful for indirect detection of changes in bone metabolism and mineral homeostasis at the level of the whole organism. Since the same measurements are routinely performed in humans, a direct comparison between the human and animal model can be made. Also, repetitive collection of blood and urine to establish a time course can be made in most laboratory animals.

There are several important limitations of biochemical markers. They provide no information regarding bone mass and strength. They do not distinguish between the appendicular and axial portions of the skeleton nor between cortical and cancellous bone. As a result, biomarkers may not detect important localized changes in bone metabolism. Finally, interpretation of biochemical markers must be made with great caution in rapidly growing animals as well as in severely osteopenic animals because changes in age and bone volume will

influence levels of biomarkers. Because of the limitations, biochemical markers are best used as an adjuvant to methods which directly evaluate bone mass and regional bone turnover.

Histomorphometry. Histology can be used to provide a two-dimensional assessment of bone mass and architecture [7,10,14,16,17,38]. The method has much greater resolution than densitometry and most alternative imaging techniques. One of the most powerful applications of histology is the use of fluorochrome labeling techniques to estimate changes in bone formation. This approach is called dynamic histomorphometry and is exquisitely sensitive because the fluorochromes act as time markers which can be used to limit the measurements to exclude bone that was formed prior to the treatment interval. Histology is the only routine method for estimating bone cell number. Osteoclast, preosteoblast, osteoblast, lining cell and osteocyte number can be measured directly in histological sections. Changes in osteoblast activity can be estimated from measurements of cell number and dynamic measurements. There is no well-established comparable dynamic index for bone resorption but changes in the rate of bone resorption and osteoclast activity can be inferred from the net change in osteoclast number, bone volume, and osteoblast activity. In some cases, it is possible to estimate changes in the rate of bone resorption by measuring retention of a fluorochrome label [38].

Histomorphometry has important limitations that are related to the small amount of tissue measured. Most histomorphometric measurements are normalized to a tissue sampling area. This approach is only valid when the sampling site is comparable in all of the groups. This requirement is difficult to accomplish when comparing animals of differing ages or growth rates. Histological changes at one sampling site should not be extrapolated to other skeletal sites. Densitometry, pQCT, μ CT, and RNA analysis are methods that complement bone histomorphometry, for evaluating bone mass, architecture and cell activity, respectively [25,34–39].

Histomorphometry can be performed at essentially any skeletal site and theoretically information on global changes to the skeleton could be obtained using this method. However, tissue preparation and analysis is sufficiently time consuming that for practical reasons histomorphometry is limited to evaluation of a relatively small number of sites. Most investigations focus on hindlimb long bones or lumbar vertebrae, which are representative of the appendicular and axial skeleton, respectively.

Molecular histomorphometry. Molecular histomorphometry couples the ability of conventional histology to

resolve individual cells within tissue sections and molecular techniques to detect the presence of specific molecules within a cell. Initial studies in molecular histomorphometry involved the localization of radiolabeled amino acids and nucleotides by autoradiography. Immunohistochemistry and *in situ* hybridization provide important new tools for localizing gene products to specific cells.

Mechanical Testing. Bone strength is rarely measured in humans but is assumed to be an important risk factor for osteoporotic fractures. A relationship between bone strength and fracture risk has not been established in animal models because of low fracture rates. Nevertheless, measurement of bone mechanical properties is an important tool for evaluating the functional significance of changes in bone mass and/or architecture.

Three-point bending, four-point bending and torsion testing are the most common methods of measuring bone mechanical properties. These measurements are performed at the midshaft diaphysis, a site at which osteoporotic fractures are uncommon. Compression testing of vertebrae and cantilever testing of the head of the femur have been developed. These newer techniques are highly recommended because they more closely approximate the type of failures associated with osteoporotic fractures [40].

Fracture Repair. Well-characterized animal models for fracture healing have been developed [41], but fracture repair studies are not routinely performed in osteoporotic animal models. Such studies are urgently needed because impaired fracture repair can dramatically increase morbidity in elderly patients. Animal models can be used to investigate the effects of age, hormones and life-style choices on fracture repair. Existing and future treatments may significantly reduce the risk of osteoporotic fractures but there is no immediate likelihood that intervention will completely prevent fractures. It is therefore imperative that potentially therapeutic interventions for osteoporosis be carefully investigated in animal models to also evaluate its effect on fracture healing.

Cell Biology. Animal models for osteoporosis can provide a source of cells for *in vitro* studies designed to evaluate potential changes in the composition and/or proliferative capacity of bone cell populations. Isolated cells can also be profitably used for studies of disturbed signaling pathways.

Summary

Animal models will continue to be important tools in the quest to understand the contribution of specific genes to

establishment of peak bone mass and optimal bone architecture, as well as the genetic basis for a predisposition toward accelerated bone loss in the presence of co-morbidity factors such as estrogen deficiency. Existing animal models will continue to be useful for modeling changes in bone metabolism and architecture induced by well-defined local and systemic factors. However, there is a critical unfulfilled need to develop and validate better animal models to allow fruitful investigation of the interaction of the multitude of factors which precipitate senile osteoporosis.

Well characterized and validated animal models that can be recommended for investigation of the etiology, prevention and treatment of several forms of osteoporosis have been listed in Table 1. Also listed are models which are provisionally recommended. These latter models have potential but are inadequately characterized, deviate significantly from the human response, require careful choice of strain or age, or are not practical for most investigators to adopt. It cannot be stressed strongly enough that the enormous potential of laboratory animals as models for osteoporosis can only be realized if great care is taken in the choice of an appropriate species, age, experimental design, and measurements. Poor choices will result in misinterpretation of results which ultimately can bring harm to patients who suffer from osteoporosis by delaying advancement of knowledge.

Abbreviations

AOB—osteoblast activity
 BMC—bone mineral content
 BMD—bone mineral density
 BMP—bone morphogenetic protein
 Cbfa1—core binding factor A1
 CSF 1—colony-stimulating factor 1
 FGF—fibroblast growth factor
 fos—transcription factor fos
 HTLV—human T cell leukemia virus
 IL—interleukin
 jun—transcription factor jun
 LHRH—luteinizing hormone releasing hormone
 Npt2—renal-specific sodium phosphate cotransporter
 NFκB—transcription factor, nuclear transcription factor
 kappa-B
 NOc—osteoclast number
 NOb—osteoblast number
 OVX—ovariectomy
 ORX—orchiectomy
 oim—osteogenesis imperfecta
 PTH-PTHrP—parathyroid hormone—parathyroid hormone related protein

RANKL—receptor activator of NF κ B ligand
 SHAM—sham-operated
 Pu.1—hematopoietic transcription factor
 TGF β —transforming growth factor β
 TNF—tumor necrosis factor
 TRAF6—TNF receptor-associated factor 6

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The Dose-Response Effects of Ethanol on the Human Fetal Osteoblastic Cell Line

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ABSTRACT

Alcohol is a risk factor for the development of osteoporosis, especially in men. Chronic alcohol abuse decreases bone mass, which contributes to the increased incidence of fractures. To better understand the mechanism of action of ethanol on bone metabolism, we have studied the dose-response effects of ethanol on conditionally immortalized human fetal osteoblasts (hFOB) in culture. Ethanol treatment had no significant effects on osteoblast number after 1 day or 7 days. Ethanol treatment did not reduce type I collagen protein levels at either time point at any dose but slightly reduced alkaline phosphatase activity after 7 days. The messenger RNA (mRNA) levels for alkaline phosphatase, type I collagen, and osteonectin were unaltered by 24 h of ethanol treatment but a high dose (200 mM) reduced mRNA levels for the two bone matrix proteins after 7 days. Ethanol treatment led to dose-dependent increases in transforming growth factor $\beta 1$ (TGF- $\beta 1$) mRNA levels and decreases in TGF- $\beta 2$ mRNA levels. The concentration of ethanol in the medium decreased with time because of evaporation but there was little degradation caused by metabolism. These results, which show that cultured osteoblasts are less sensitive than osteoblasts in vivo, suggest that the pronounced inhibitory effects of ethanol on bone formation are not caused by direct cell toxicity. (J Bone Miner Res 2001;16:270–276)

Key words: alcohol abuse, bone formation, osteoporosis, bone fractures

INTRODUCTION

CHRONIC ALCOHOL abuse is associated with pronounced detrimental effects on the musculoskeletal system. Numerous reports implicate alcohol as a major risk factor for osteoporosis, especially in men. Habitual alcohol abuse clearly results in bone loss while moderate intake of alcohol has been reported to have variable effects on bone mass, depending on age and gender.⁽¹⁾ However, the mechanism for the bone loss induced by alcohol is not understood. There is consensus that alcohol abuse results in decreased bone formation.^(2,3) On the other hand, the effects of alcohol on bone resorption are less certain with a number of conflicting reports.^(4–9)

Alcohol results in dose-dependent decreases in rat bone formation with dose rates comparable with alcoholics, lead-

ing to osteopenia.^(10–13) The molecular mechanism(s) that mediates this bone loss is characterized poorly. Some studies suggest that ethanol has a direct toxic effect on osteoblasts.⁽¹⁴⁾ The histological changes to bone are preceded by changes in messenger RNA (mRNA) levels for bone matrix proteins and cytokines.⁽¹²⁾ Rapid (within 6 h) transient dose-dependent changes in expression of matrix proteins in bone have been reported in the rat, suggesting that exposure to ethanol results in reversible changes in osteoblast function. Farley et al. have shown that chemicals, including ethanol, modify membrane fluidity and alter the response of bone cells to mitogens.⁽¹⁵⁾ This suggests that some of the actions of ethanol in vivo may be influenced by systemic factors, including gonadal hormones and cytokines. To better understand the mechanism of ethanol action on skeletal development, we have studied the direct effects of ethanol on

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immortalized human fetal osteoblast (hFOB) cells⁽¹⁶⁾ in culture. In these studies, hFOB cells were exposed to ethanol at concentrations corresponding to blood alcohol levels relevant to moderate drinking (10 mM; 0.046% wt/vol) and chronic alcohol abuse (50 mM; 0.23% wt/vol), as well as concentrations incompatible with human life (≥ 100 mM; 0.46% wt/vol).

MATERIALS AND METHODS

Cell culture and ethanol treatment

The hFOB cell line that contains the temperature-sensitive T-antigen expression vector with the neomycin resistance gene⁽¹⁶⁾ was maintained at 34°C in phenol red-free Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% charcoal-stripped fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO, USA) and supplemented with geneticin (300 μ g/ml; Gibco/BRL, Rockville, MD, USA). The cells were plated into T-75 flasks at 1×10^6 cells per flask 24 h before ethanol treatment. The cells were treated with ethanol (10–500 mM) for 1 day and 7 days. During 7-day treatment, the media were changed with fresh media containing ethanol on day 4 and maintained for an additional 3 days. Cells were washed with phosphate-buffered saline (PBS) and harvested at the end of ethanol treatment for RNA analyses. The media were stored and used for type I collagen protein analyses.

Ethanol assay

Ethanol content in media was determined using a kit as per the manufacturer's protocol (Sigma Chemical Co., St. Louis, MO, USA).

RNA isolation

Total cellular RNA was extracted and isolated using a modified organic solvent method and the RNA yields were determined spectrophotometrically at 260 nm.⁽¹⁷⁾

Northern blot hybridization

Ten micrograms of each sample was denatured by incubation at 52°C in a solution of 1 M glyoxal, 50% dimethyl sulfoxide, and 0.01 M NaH_2PO_4 and then separated in a 1% agarose gel. The amounts of RNA loaded and transferred were assessed by methylene blue staining of the membranes and hybridization with a [^{32}P]-labeled complementary DNA (cDNA) for 18S ribosomal RNA (rRNA). RNA that was separated in agarose gels was transferred to an Amersham Hybond nylon membrane (Amersham, Arlington Heights, IL, USA) overnight via capillary action in $20\times$ SSC ($1\times$ SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) sodium citrate buffer. The membranes were baked in a vacuum oven at 80°C for 2 h before hybridization. Membranes were prehybridized for 2 h at 45°C in buffer containing 50% deionized formamide, 10% dextran sulfate, $5\times$ SSC, 100 μ g/ml of heat-denatured single-strand salmon sperm DNA, and $2\times$ Denhardt's solution. Hybridization was conducted for 80 minutes in a buffer containing the

previously mentioned ingredients in addition to a minimum of 1×10^6 cpm/ml [^{32}P]-labeled cDNA probe. Labeled cDNAs for alkaline phosphatase, osteocalcin, and type I collagen were used as probes. cDNA probes were labeled by random sequence hexanucleotide primer extension using the Megaprime DNA labeling kit from Amersham. Membranes were washed for 30 minutes at 65°C in $2\times$ SSC and for 15–60 minutes in $0.1\times$ SSC at 45°C. The resulting radioactive mRNA bands on the blots were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and normalized to 18S rRNA.

The cDNA probes were: (1) alkaline phosphatase cDNA (a gift from Dr. Gideon Rodan, Merck, Sharp, and Dohme, West Point, PA, USA); (2) type I collagen cDNA probe obtained from Lofstrand Labs, Ltd. (Gaithersburg, MD, USA)⁽¹⁸⁾; (3) osteonectin cDNA (a gift from Dr. G. Long, University of Vermont, Burlington, VT, USA)⁽¹⁹⁾; and (4) cDNA for 18S rRNA was purchased from Ambion (Austin, TX, USA).

RNAse protection assay for cytokines

We measured the mRNA concentrations of transforming growth factor $\beta 1$ (TGF- $\beta 1$), TGF- $\beta 2$, and TGF- $\beta 3$; tumor necrosis factor α (TNF- α) and TNF- β ; interleukin-1 α (IL-1 α), IL-1 β , IL-1Ra, IL-6, IL-10, and IL-12 (p35 and p40); interferon β (IFN- β) and IFN- γ ; and lymphotoxin β (LT- β) by RNAse protection assays. Quantitation of protected RNA fragments was performed by PhosphorImager analyses and normalized to glyceraldehyde-3-phosphate dehydrogenase and ribosomal structural protein L32.

Type I collagen protein assay

The medium from ethanol-treated hFOB cells was collected at the end of 24 h and 7 days of ethanol treatment, centrifuged to remove debris before used for type I procollagen assays. The type I procollagen assay, which measures the propeptide portion of the molecule, reflects the synthesis of the mature form of the protein and it was carried out using Prolagen-C kit as described by the manufacturer's protocol (Metra Biosystems, Mountainview, CA, USA). The type I procollagen levels obtained were normalized to total protein concentrations that were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

Alkaline phosphatase activity

The alkaline phosphatase activity was measured using a kit (Sigma Chemical Co.). The cells treated with ethanol were rinsed with PBS, harvested, and processed for determining the alkaline phosphatase activity. The activity was normalized to total cellular protein, which was determined by BCA protein assay.

Statistical analysis

In each experiment, three to six replicates of each treatment were measured. Unless indicated otherwise, the data represent the mean \pm SE of three independent experiments.

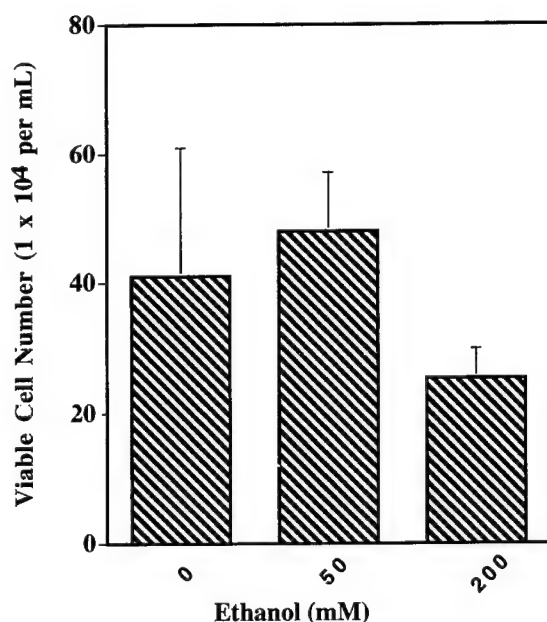


FIG. 1. Effect of 7 days of treatment of ethanol on growth of hFOB cells. Cells were treated with 0, 50, and 200 mM concentrations of ethanol. The data represent the mean \pm SE of three independent experiments. No significant changes were detected.

Significant differences between groups were determined by Fisher's protected least significant difference post hoc test for multiple group comparisons after detection of significance by one-way analysis of variance (ANOVA). Significance was considered at values of $p < 0.05$. Dose-response effects were analyzed by linear regression analysis.

RESULTS

Effect of ethanol on cell growth

The effect of ethanol treatment on hFOB cell growth was determined after 1-day and 1-week treatment with 0, 50, and 200 mM concentrations of ethanol. The viable cell numbers were measured by trypan blue dye exclusion assay in a hemocytometer. There were no changes in cell number after either 1 day (data not shown) or 1 week of treatment (Fig. 1).

Assay for alcohol levels

The amount of ethanol present in the media as a function of time after incubation at 34°C was determined (Fig. 2). The ethanol disappearance curves follow the same pattern in the presence and absence of cells. About 24% of the initial amount was still detectable after 4 days of incubation, the time at which replacement of new media containing ethanol was performed.

Effect of ethanol on bone matrix gene expression in cultured human osteoblasts

The dose (0–200 mM) effects of 24 h of ethanol treatment on alkaline phosphatase, osteonectin, and type 1 col-

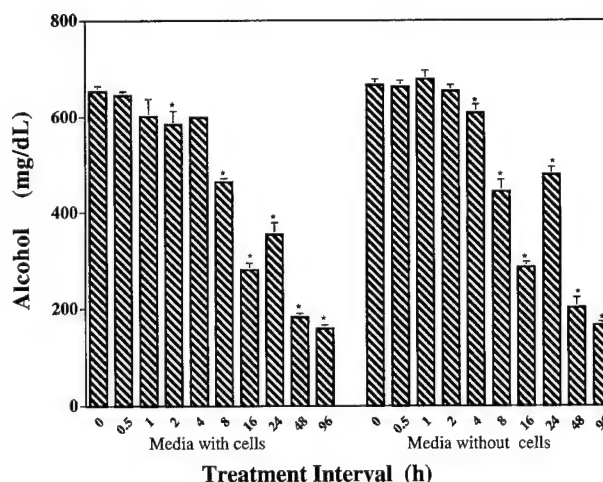


FIG. 2. Time course for ethanol present in the media. Culture dishes containing media in the presence and absence of hFOB cells were treated with 200-mM concentrations of ethanol. The data represent the mean \pm SE of three independent experiments. $*p \leq 0.05$ (compared with untreated control, by one-way ANOVA and Fisher's projected least significant difference [PLSD] analysis).

lagen mRNA levels studied by Northern analysis in hFOB cells in culture are shown in Fig. 3. Ethanol had no effect on steady-state mRNA levels for these markers of osteoblast differentiation and activity.

The dose (0–200 mM) effects of 7 days of ethanol treatment on alkaline phosphatase, type I collagen, and osteonectin mRNA levels are shown in Fig. 4. Ethanol had no effect on the mRNA levels for alkaline phosphatase. The type I collagen mRNA levels were significantly decreased by 46% at 200 mM. Finally, ethanol decreased the mRNA levels for osteonectin by 31% at 200 mM.

The effects of (0–200 mM) ethanol on type I collagen protein levels after 24 h and 1 week of ethanol treatment are shown in Fig. 5. The type I collagen protein levels did not change significantly after 24 h of ethanol treatment in hFOB cells in culture, whereas after 1 week, it significantly increased by 57% at 50 mM of ethanol (Fig. 5).

To determine whether the alcohol alters alkaline phosphatase activity, we measured the activity at two different doses of ethanol (50 mM and 200 mM) after 24 h and 1 week (Fig. 6). The alkaline phosphatase activity was not significantly changed at either dose after 24 h. After 1 week of treatment, the alkaline phosphatase activity was decreased at both doses of ethanol.

Effect of ethanol on cytokines and growth factors

We studied the changes in steady-state mRNA levels for selected cytokines that have been implicated in the regulation of bone formation and resorption. We analyzed the mRNA concentrations for members of the IL family, IFNs, TNF, and TGF- β by RNase protection assay (Table 1). Although many cytokine genes were not detectable by our assay (Table 1), there were changes in the mRNA levels of TGF- β 1 and TGF- β 2 (Fig. 7). The mRNA levels of TGF- β 1 increased whereas that of TGF- β 2 decreased with

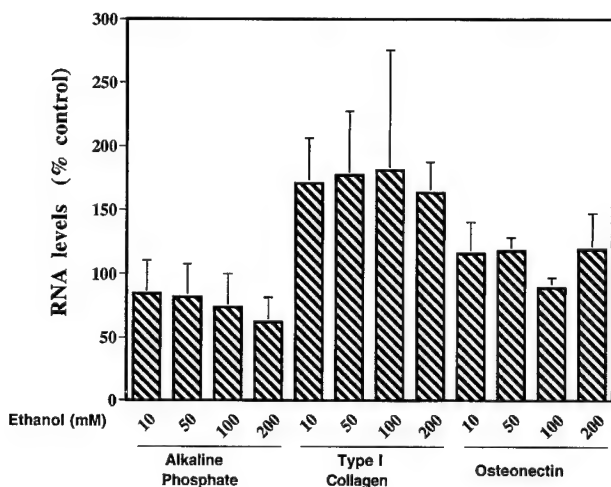


FIG. 3. Effect of ethanol at various doses after 1-day treatment on alkaline phosphatase, type 1 collagen, and osteonectin mRNA levels. Total RNA was isolated from cells treated with 0-, 10-, 50-, 100-, and 200-mM concentrations of ethanol and analyzed by Northern blot hybridization using specific cDNA probes. The radioactive signal measured by PhosphorImager analysis has been expressed as a percentage of the value obtained from untreated controls. The data represent the mean \pm SE of three independent experiments. No significant changes were detected.

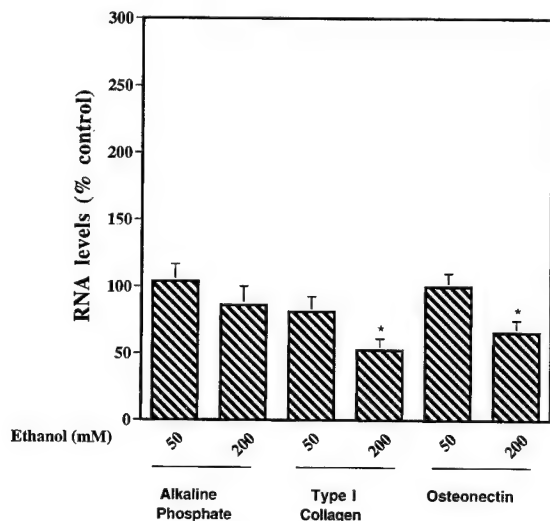


FIG. 4. Effect of ethanol at various doses after 1 week of treatment on alkaline phosphatase, type 1 collagen, and osteonectin mRNA levels. Total RNA was isolated from cells treated with 0-, 50-, and 200-mM concentrations of ethanol and analyzed by Northern blot hybridization using specific cDNA probes. The radioactive signal measured by PhosphorImager analysis has been expressed as a percentage of the value obtained from untreated controls. The data represent the mean \pm SE of three independent experiments. * $p \leq 0.05$ (compared with untreated control, by one-way ANOVA and Fisher's projected least significant difference [PLSD] analysis).

increasing ethanol concentrations (Figs. 7B and 7C). Linear regression analysis revealed significant dose relationships for TGF- β 1 ($R = 0.81$; $p < 0.05$) and TGF- β 2 ($R = -0.50$; $p < 0.05$). TGF- β 3 mRNA was not detected by this assay both in control and in ethanol-treated cells.

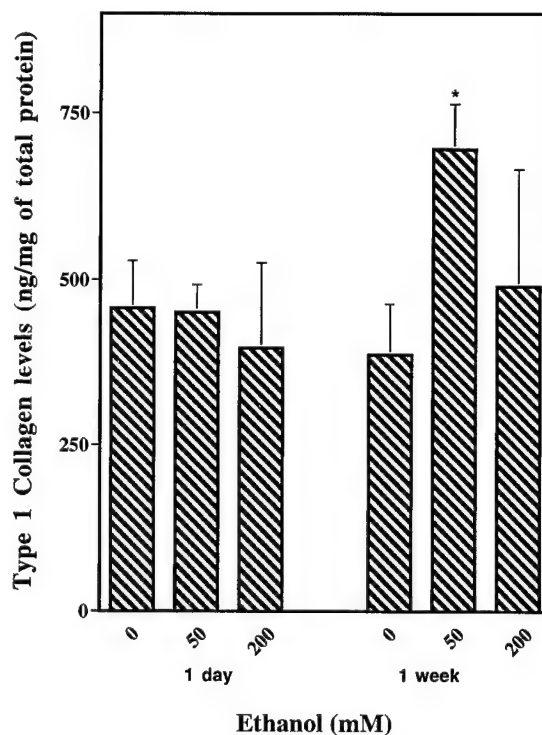


FIG. 5. Effects of 1-day and 1-week treatments of ethanol on type 1 collagen protein levels in hFOB cells. Cells were treated with 0-, 50-, and 200-mM concentrations of ethanol and the type 1 collagen levels in the medium were measured. The results represent the mean \pm SE of three independent experiments. * $p \leq 0.05$ (compared with untreated control, by one-way ANOVA and Fisher's projected least significant difference [PLSD] analysis).

DISCUSSION

Although alcohol abuse has been long associated with osteoporosis, uncovering the mechanism of ethanol-mediated bone loss has been a challenging task. Interpretation of human data is difficult because of the uncertainties that arise as a result of the many problems inherent to performing controlled experiments in alcoholics. Nevertheless, a consensus has arisen that alcohol inhibits bone formation in humans.⁽²⁾ Studies in laboratory animal models for alcohol abuse, which are much easier to control than human studies, have confirmed that ethanol inhibits bone growth, decreases bone formation, and leads to osteopenia.^(11,13,20) However, these animal studies cannot distinguish a direct toxic effect of ethanol on osteoblasts from alternative indirect mechanisms such as a detrimental response to more toxic metabolites, changes in circulating levels of hormones and other systemic factors, and local production of cytokines and other signaling molecules. The present dose-response study using cultured normal hFOB⁽¹⁶⁾ was designed to determine whether ethanol has a direct toxic effect on osteoblast growth and gene expression. This hFOB cell line has been well characterized and has been shown to respond to known regulators of osteoblast activity and gene expression.⁽²¹⁻²⁵⁾ The results show that ethanol has

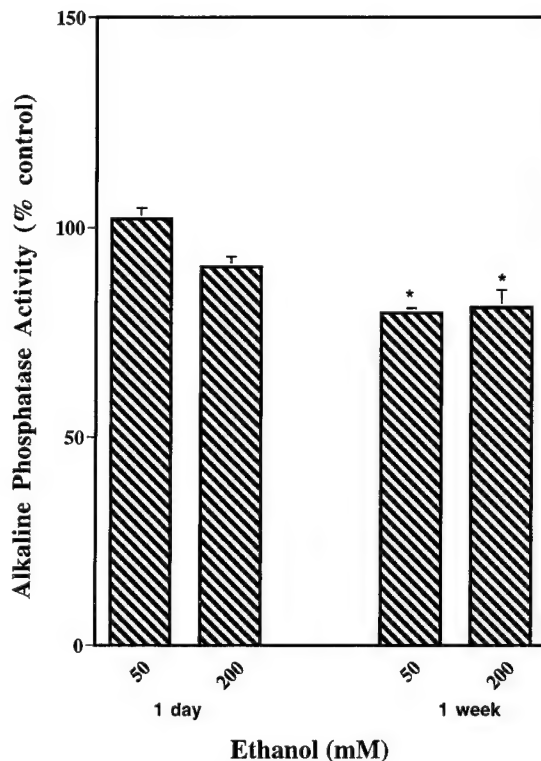


FIG. 6. Effects of ethanol treatment on alkaline phosphatase activity. The hFOB cells treated with 0-, 50-, and 200-mM concentrations of ethanol for 1 day and 1 week were harvested and the alkaline phosphatase activity in the cell pellet was measured. The results represent the mean \pm SE of six replicate cultures from a single experiment. * $p \leq 0.05$ (compared with untreated control, by one way ANOVA and Fisher's projected least significant difference [PLSD] analysis).

TABLE 1. LIST OF CYTOKINES ANALYZED BY RNASE PROTECTION ASSAY IN ETHANOL-TREATED hFOB CELLS

IL-12 p35	Not detected
IL-12 p40	Not detected
IL-10	Not detected
IL-6	Not detected
IL-1 α	Not detected
IL-1 β	Not detected
IL-1Ra	Not detected
IFN- β	Not detected
IFN- γ	Not detected
LT- β	Not detected
TNF- α	Not detected
TNF- β	Not detected
TGF- β 1	Increased
TGF- β 2	Decreased
TGF- β 3	Not detected

mixed effects on indices of osteoblast differentiation and no significant effect on osteoblast number. These generally weak effects contrast to the robust response to alcohol observed in humans and laboratory animals at much lower concentrations of ethanol.

Blood alcohol levels of <20 mM are sufficient to reduce biochemical markers of bone formation in humans rapidly,

as well as histological indices of bone formation in rats. Indeed, bone formation was significantly decreased in rats fed alcohol in their diet comprising as little as 3% of total caloric intake resulting in blood alcohol levels below 5 mM (R.T. Turner, unpublished data). On the other hand, our present study shows that a wide range of ethanol concentration (10–200 mM) had no short-term (1 day) effect on indices of osteoblast differentiation (alkaline phosphatase, osteonectin, and collagen mRNA levels). Additionally, alcohol had no detrimental effect on short- or long-term (7 days) accumulation of collagen in the media and the alkaline phosphatase activity was only slightly reduced after a long duration of ethanol treatment. These results provide strong evidence that ethanol has minimal toxic effects on the mature osteoblast. We cannot rule out the possibility that hFOB cells are more resistant to the direct toxic effects of alcohol than osteoblasts in vivo. However, if this were the case, it would represent a general in vitro phenomenon because other osteoblastic cell lines are similarly resistant to ethanol.⁽²⁶⁾

The lack of short-term effects of ethanol on expression of bone matrix protein genes in cultured osteoblasts contrasts with the dramatic in vivo response. Ethanol has transient tissue and dose-dependent effects on steady-state mRNA levels for extracellular matrix proteins (collagen, osteocalcin, and osteonectin) in rats.⁽¹²⁾ Acute ethanol treatment resulted in a coordinated increase in mRNA levels for all the matrix proteins in bone but not in the uterus and liver. However, continued treatment resulted in a decrease in mRNA levels for the matrix proteins as well as a decrease in bone formation.⁽¹²⁾ The results obtained in the present studies suggest that the changes in gene expression observed in vivo are not caused by a direct effect of ethanol on the mature osteoblast. The relatively small inhibitory effects of very high concentrations of ethanol on mRNA levels for matrix proteins after prolonged exposure may have been mediated by the observed changes in TGF- β expression. The physiological significance of this proposed mechanism can be questioned because the changes in matrix protein gene expression were observed only at levels of ethanol unlikely to be observed in humans. Nevertheless, the apparent linear dose responses of TGF- β 1 and TGF- β 2 steady-state mRNA levels suggest that expression of these cytokines also might be influenced by a lower concentration of ethanol. However, there is no in vivo data to support changes in TGF- β expression in bone with alcohol.

With the exception of TGF- β 1 and TGF- β 2, we either could not detect or did not observe changes in gene expression of any of the other cytokines analyzed. TGF- β is an important signaling protein produced by osteoblasts that affects both bone formation and bone resorption.⁽²⁷⁾ It promotes osteoclast apoptosis but inhibits osteoblast apoptosis, thus exerting an influence on bone remodeling. Overexpression of TGF- β 2 in transgenic mice deregulates bone remodeling, leading to an age-dependent loss of bone mass that resembles high-turnover osteoporosis in humans.⁽²⁸⁾ These mice also have an increased rate of bone matrix formation, which is not because of direct action of TGF- β but because of homeostatic response to the increase in bone resorption caused by TGF- β . These observations confirm that TGF- β is a physiological regulator of bone metabolism, suggesting

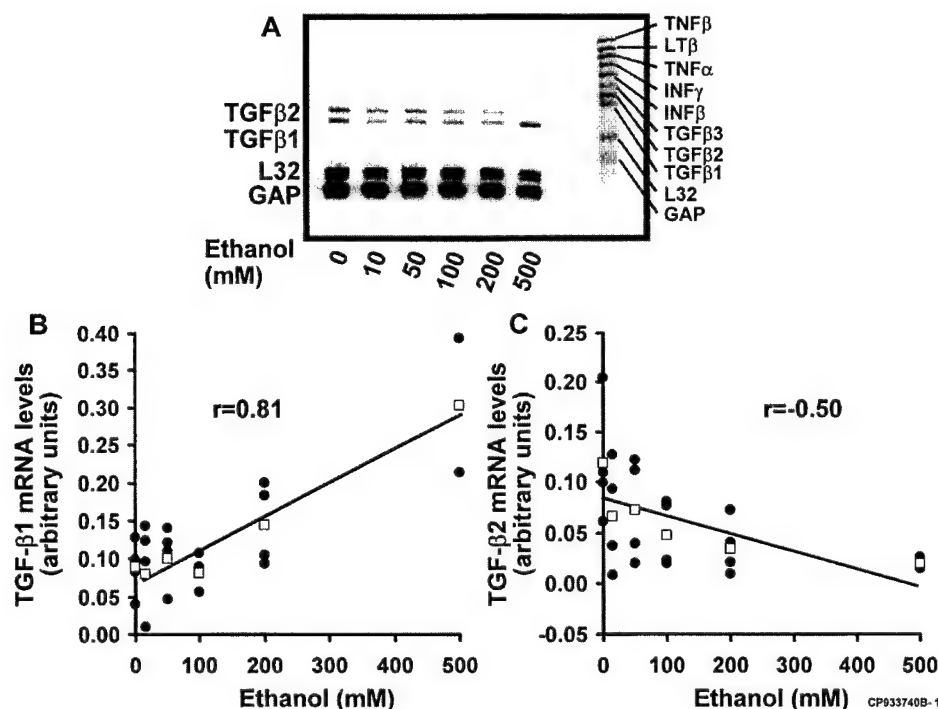


FIG. 7. Effect of 1-day ethanol treatment on TGF- β mRNA levels. Total RNA (10 μ g) isolated from ethanol-untreated and -treated cells was analyzed by ribonuclease protection assay. (A) The radioactive signals from the gel were measured by PhosphorImager analysis. The data points in closed circles (●) are mRNA levels per ethanol dose for (B) TGF- β 1 and (C) TGF- β 2. The open square (□) represents the mean for each dose. The mRNA response of TGF- β 1 and TGF- β 2 correlate significantly with ethanol doses ($p \leq 0.002$ and $p \leq 0.017$, respectively).

that it may have a role in mediating some of the effects of alcohol. The observed alterations in TGF- β by alcohol are not unique to osteoblasts. Ethanol-mediated induction of TGF- β has been reported in macrophages.⁽²⁹⁾ Also, ethanol at physiologically relevant concentrations (25 mM) has been shown to induce TGF- β in monocytes.⁽³⁰⁾

The cells in our study were not exposed to uniform concentrations of ethanol; there was a gradual decrease with time until the media were replaced, which restored ethanol levels to the previous maximal concentrations. This pulsatile exposure, although imperfect, is likely to model more accurately the changes in blood levels in chronic drinkers than maintenance of constant levels of alcohol. The nearly identical time-course changes in ethanol concentration in culture dishes with and without cells suggest that hFOB cells are unable to metabolize ethanol. This finding is significant because the metabolites of ethanol may be more toxic than the parent compound.^(31,32)

A direct inhibition of osteoblast proliferation is supported by the work of Klein et al.⁽²⁶⁾ who have shown that ethanol decreases [3 H]thymidine incorporation and reduces cell growth in cultured TE85 osteosarcoma cells. Our observed tendency for a reduction in cell number generally is consistent with the earlier result, but only at concentrations of ethanol unlikely to be observed in humans (200 mM). Further studies will be necessary to determine the respective mechanisms of the cell-specific ethanol-induced growth arrest in the two cell lines as well as the relevance for this response to the whole organism.

Finally, ethanol may have minimal effects on expression of osteoblasts in their basal state but may alter the response of these cells to external regulatory signals. Bone turnover is regulated by locally produced as well as systemic factors.^(27,33-36) Disruption of one or more of these important signaling pathways could have detrimental effects on

bone formation. This possibility is supported by Farley et al., who have shown that ethanol disrupts the response of primary cultures on bone cells to several mitogenic agents.⁽¹⁵⁾ hFOB cells have not been shown to express mRNA for either insulin-like growth factor I (IGF-I) or TNF- α . This may be important to the failure to detect large effects of ethanol on these cells because those two cytokines are clearly altered in skeletal tissues in vivo.⁽¹²⁾ Whatever the specific mechanism by which alcohol indirectly inhibits bone formation, our finding that ethanol does not have a toxic effect on the osteoblast is important because it increases the likelihood that the detrimental effects of ethanol on osteoblast functions are reversible.

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Effects of Parathyroid Hormone on Bone Formation in a Rat Model for Chronic Alcohol Abuse

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Background: Alcoholism is a risk factor for osteoporosis and it is not clear whether the detrimental effects of alcohol on bone are reversible. Parathyroid hormone (PTH) is a potent stimulator of bone matrix synthesis and is being investigated as a therapeutic agent to reverse bone loss. The present investigation was designed to determine the effects of PTH on bone formation in a rat model for chronic alcohol abuse.

Methods and Results: Alcohol was administered in the diet of female rats (35% caloric intake) for 2 weeks. Human (1-34) PTH (80 $\mu\text{g/kg/day}$) was administered subcutaneously during the second week of the study. Alcohol resulted in a transient reduction in steady-state mRNA levels for the bone matrix proteins type 1 collagen, osteocalcin, and osteonectin compared with rats that were fed an alcohol-free (control) diet. As expected, alcohol decreased and PTH increased histologic indices of bone formation. Additionally, two-way ANOVA demonstrated that alcohol antagonized PTH-induced bone formation. Despite antagonism, bone formation and mRNA levels for bone matrix proteins in alcohol-fed rats treated with PTH greatly exceeded the values in rats fed the control diet.

Conclusions: The results of this study contribute to a growing body of evidence that alcohol-induced bone loss is primarily due to reduced bone formation. We conclude that alcohol does not prevent the stimulatory effects of PTH on bone formation. This is evidence that the effects of alcohol on the skeleton are reversible. Additionally, the positive effects on bone formation in rats that consumed high concentrations of alcohol suggested that PTH may be useful as an intervention to treat alcohol-induced osteoporosis.

Key Words: Alcoholism, Osteoblasts, Osteoclasts, Bone Histomorphometry, Bone Resorption.

ALCOHOLICS OFTEN HAVE radiographic evidence of osteopenia, a greatly reduced bone mineral density, and reduced histologic and biochemical indices of bone formation (Bikle et al., 1985, 1993; Crilly et al., 1988; Diez et al., 1994; Gonzalez-Calvin et al., 1993; Harding et al., 1988; Labib et al., 1989; Laitinen et al., 1992, 1993; Lalor et al., 1986; Nielsen et al., 1990; Odvina et al., 1995; Pumarino et al., 1996; Schnitzler and Solomon, 1984; Spencer et al., 1986). There is also evidence that alcoholics are at a greater risk for receiving fractures than healthy individuals (Kanis et al., 1999; Kelepouris et al., 1995). Alcohol-induced osteoporosis differs from postmenopausal bone loss in that the pronounced increase in bone turnover that follows the menopause is not observed in alcoholics (Felson et al., 1993; Heaney et al., 1978). The principal mechanism for alcohol-induced bone loss appears to be reduced bone formation (Bikle et al., 1993; Gonzalez-Calvin et al., 1993; Labib et al., 1989; Laitinen et al., 1992; Nielsen et

al., 1990; Schnitzler and Solomon, 1984); indices of bone resorption may be increased, decreased, or unchanged (Bikle et al., 1985, 1993; Crilly et al., 1988; Diez et al., 1994; Laitinen et al., 1991a, 1994; Lalor et al., 1986; Schnitzler and Solomon, 1984). The net reduction in bone formation appears to result in a negative remodeling balance and ultimately to osteopenia (Turner, 2000).

At present, there is no specific intervention to treat alcoholics who have osteoporosis, but a rat model for the skeletal effects of alcohol abuse has been established to investigate possible therapies. Rats fed ethanol at a rate (adjusted for the difference in body mass) comparable to alcoholics develop abnormalities in bone and mineral homeostasis similar to alcoholics (Peng et al., 1972; Peng and Gitelman, 1974; Sampson, 1998; Sampson et al., 1997; Turner et al., 1987, 1988, 1991). These abnormalities include osteopenia and a pronounced inhibition of bone formation (Sampson, 1998; Sampson et al., 1997; Turner et al., 1987, 1988, 1991).

Parathyroid hormone (PTH) is under investigation for treatment of osteoporosis. Pulsatile administration of PTH increases bone formation and bone mass in humans and laboratory animals (Dobnig and Turner, 1995). The principle purpose of the present study was to evaluate the efficacy of PTH as a therapy to reverse the inhibitory effects of alcohol abuse on bone formation in the rat model.

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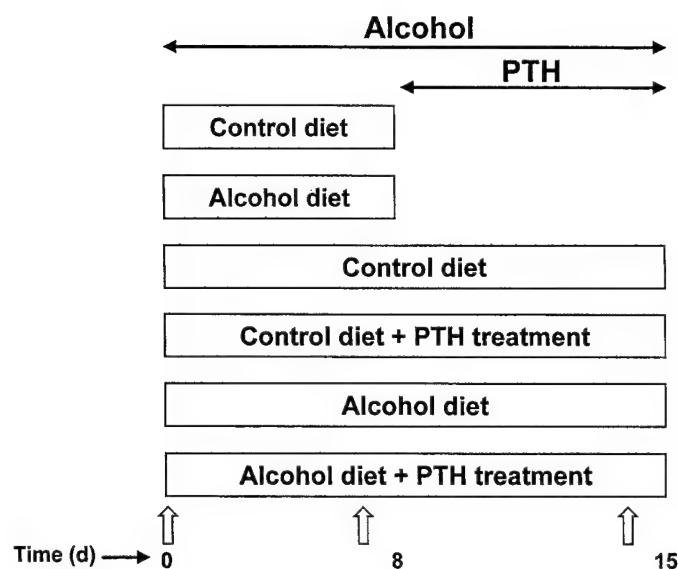


Fig. 1. A schematic showing the experimental design. Alcohol treatment was started on day 0 and PTH treatment was started on day 8. Fluorochrome labels (↑) were administered on day 0, 7, and 14. The baseline control groups were killed on day 8 and the remaining groups on day 15.

MATERIALS AND METHODS

Animals

Six-month-old female Sprague Dawley® rats (Harlan, Sprague Dawley, Indianapolis, IN) weighing an average of 294 ± 2 g (mean \pm SE) were randomly assigned to one of six groups ($n = 10$ rats/group). All animal procedures were approved by the Institutional Animal Welfare Committee. Rats were individually housed in a temperature- and humidity-controlled animal facility on a 12-hr light/dark cycle. All animals were acclimated to a modified Lieber-DeCarli liquid diet (BioServe, Frenchtown, NJ) for one week. The three alcohol-supplemented groups were subsequently fed, ad libitum, a liquid diet containing increasing concentrations of ethanol (95% v/v) until they received 35% of total caloric intake as alcohol at the end of the first treatment week (Kidder and Turner, 1998). This dose of alcohol results in blood alcohol levels and changes in bone and mineral metabolism similar to alcoholics (Kidder and Turner, 1998; Sampson, 1998; Sampson et al., 1997; Turner et al., 1987, 1988, 1991). Rats were not given water. The three control diet groups did not receive ethanol and were fed the same liquid diet isocalorically supplemented with maltose/dextran, as per the manufacturer's instructions. All animal weights were recorded on the first day of study (day 1), on the first day of treatment with PTH (day 8), and on the day of necropsy (day 15). The 24-hr consumptions were recorded daily and the rats that were fed the control diet were pair-fed to the mean consumption of the alcohol-treated animals.

A schematic of the experimental design is shown in Fig. 1. On the first day of ethanol treatment, all rats were injected with a 20 mg/kg BW dose of a bone fluorochrome, tetracycline-hydrochloride (Sigma, St. Louis, MO), perivascularly at the base of the tail. All rats were injected 7 days later with another bone fluorochrome, calcein (20 mg/kg BW; Sigma). One group of rats that were fed alcohol and one group of rats that were fed the control diet were euthanized on day 8 to provide baseline measurements for the PTH intervention study.

Starting on day 8, two groups of rats (one fed the control diet and one fed the alcohol diet) were administered human PTH (1–34) daily (80 μ g/kg) subcutaneously, as described (Dobnig and Turner, 1997). Also, starting on day 8, the remaining two groups of rats (one control diet and one alcohol diet) were given carrier only. All remaining rats were given the fluorochrome demeclocycline (20 mg/kg BW; Sigma) on day 14 and euthanized on day 15.

Rats were anesthetized using ketamine (100 mg/ml) and xylazine (100 mg/ml) and killed by decapitation. Tibiae were defleshed and either frozen in liquid nitrogen and stored at -80°C prior to RNA analysis or fixed by immersion in 70% ethanol for bone histomorphometry. Uteri were harvested and wet weights recorded.

Bone Histomorphometry

Tibiae were dehydrated by immersion in a series of increasing concentrations of ethanol, and embedded without demineralization in a methylmethacrylate mixture (methylmethacrylate:2-hydroxyethyl-methacrylate 12.5:1). Longitudinal sections were taken from the middle of the proximal tibia with a Reichert-Jung microtome (5 μ m thick) and permanently mounted on slides for histomorphometric measurements. Cancellous bone measurements were performed in a standard sampling site located on the long axis of the bone, one millimeter from the epiphyseal growth plate at its most distal point. This site is distal to the primary spongiosa, within the secondary spongiosa, and extends bilaterally to exclude the endocortical edges. The sampling site encompassed a tissue area of 2.88 mm².

All histomorphometric measurements were carried out with a semiautomatic image-analysis system, which consisted of a Compaq computer interfaced with a microscope and image analysis software (OsteoMetrics, Inc., Atlanta, GA). Skeletal indices were measured by registering the movement of a digitizing mouse across a graphics tablet as a tracing was superimposed on an image of the section displayed on a video screen. The computer software recorded lengths and calculated the enclosed areas. The following histomorphometric indices were determined as described (Kidder and Turner, 1998): cancellous bone volume (BV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), mineralizing surface (LS), mineral apposition rate (MAR), and bone formation rate (BFR). Measurements were performed in accordance with standardized nomenclature and methods (Parfitt et al., 1987).

Northern Hybridizations

Total RNA was isolated from tibial metaphyses for Northern analysis of steady-state mRNA levels for selective bone matrix proteins. Metaphyses from frozen tibiae were individually crushed into powder using a Spex freezer mill (Edison, NJ) and total cellular RNA was extracted and isolated with a modified organic solvent method as described (Turner et al., 1998a). Ten μ g of total RNA from each sample were denatured in 1 M glyoxal, 50% dimethylsulfoxide 0.01 M NaH₂PO₄ at 52°C, separated by electrophoresis on a 1% agarose gel, transferred by capillary action in 20X SSC to nylon membranes (Amersham Hybond nylon membrane, Arlington Heights, IL) and crosslinked using a UV source (Stratagen, LaJolla, CA) before hybridization procedures. Membranes were prehybridized for 1–2 hrs at 65°C in a Rapid-Hyb Buffer (Amersham). Hybridization was carried out for 80 min in a buffer containing the above ingredients in addition to a minimum of 4×10^6 cpm/ml ³²P-labeled cDNA for the following bone matrix proteins: prepro- α (I) subunit of type I collagen (collagen), osteocalcin, and osteonectin. The amounts of RNA loaded and transferred were assessed by methylene blue staining of the membranes and by hybridization with ³²P-labeled cDNA for 18S ribosomal RNA. Representative Northern blots of bone extracts from ethanol and from PTH-treated rats are published (Dobnig and Turner, 1997; Turner et al., 1998a).

cDNA probes were labeled by random sequence hexanucleotide primer extension using the Megaprime DNA labeling kit (Amersham). The membranes were washed for 30 min at RT in 3X SSC and for 15 min in 1X SSC at 43–65°C.

The mRNA bands on the Northern blots were quantitated by densitometric scanning (Molecular Dynamics Phosphor Imager, Sunnyvale, CA) and values normalized to 18S ribosomal RNA. Representative photographs of phosphor images of Northern blots for type 1 collagen, osteocalcin, and osteonectin are published (Turner et al., 1998a).

Statistical Analysis

Values are expressed as mean \pm SE. Group comparisons between the alcohol-treated and nonalcohol fed groups after the first week of treat-

Table 1. Effects of Alcohol and PTH on Body Weight and Uterine Weight

Group	Final body weight (g)	Uterine weight (mg)
Baseline		
Control diet	305 ± 5	865 ± 83
Alcohol diet	295 ± 6	657 ± 64
<i>t</i> -test		
Effect of Alcohol	NS	NS
Treatment		
Control Diet		
Vehicle	307 ± 5	558 ± 48
PTH	305 ± 5	637 ± 69
Alcohol Diet		
Vehicle	280 ± 5	509 ± 34
PTH	285 ± 5	496 ± 49
Two-way ANOVA		
Effect of Alcohol	NS (0.09)	NS
Effect of PTH	NS	NS
Interaction	NS	NS

Values are mean ± SE; *n* = 10.

NS, not significant.

Table 2. Effects of Alcohol on Cancellous Bone Histomorphometry After 1 Week Treatment

Measurement	Control diet	Alcohol diet	<i>p</i> Value (<i>t</i> -test)
BV/TV (%)	25.1 ± 2.9	29.2 ± 1.9	NS
Tb.Th (μm)	56.8 ± 3.2	59.7 ± 3.1	NS
Tb.N (mm ⁻¹)	4.35 ± 0.32	4.88 ± 0.19	NS
Tb.Sp (μm)	180 ± 20	147 ± 9	NS
LS/BS (%)	2.30 ± 1.6	1.40 ± 0.60	NS
BFR/BS (μm ³ /μm ² /d)	0.022 ± 0.007	0.011 ± 0.006	NS
BFR/BV (%/d)	0.080 ± 0.025	0.033 ± 0.016	NS
BFR/TV (%/d)	0.018 ± 0.004	0.011 ± 0.006	NS
MAR (μm/d)	0.94 ± 0.05	0.78 ± 0.10	NS

Values are mean ± SE; *n* = 7–8. NS is *p* > 0.05.

BV, bone volume; TV, tissue volume; LS, labeled surface; BS, bone surface; MAR, mineral apposition rate; BFR, bone formation rate; Tb, trabecular; Th, thickness; N, number; Sp, separation.

NS, not significant.

ment were determined by Student's *t* test. The respective effects of ethanol and PTH were analyzed by two-way analysis of variance. Significance was established at *p* values ≤ 0.05.

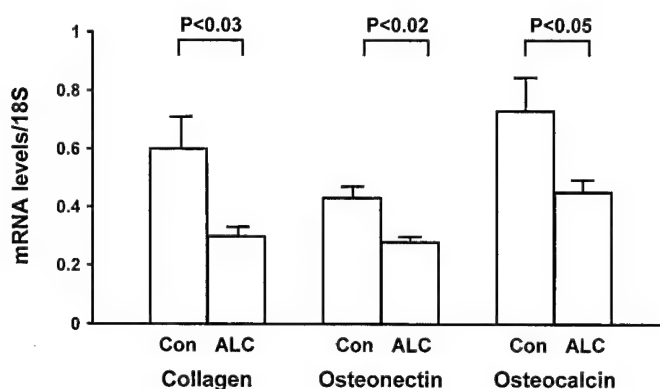
RESULTS

Body weights and uterine weights are shown in Table 1. Neither alcohol nor PTH had an effect on body weight or uterine weight.

The effects of 1 week of dietary alcohol on bone histomorphometry are shown in Table 2. Alcohol had no significant effects but there was a consistent tendency toward reduced dynamic measurements, especially LS/BS, BFR/TV, BFR/BS, and BFR/BV.

The effects of 1 week of dietary alcohol on steady-state mRNA levels for bone matrix proteins are shown in Fig. 2. Alcohol resulted in significant decreases in mRNA levels for type 1 collagen, osteonectin, and osteocalcin.

Table 3 shows the effects of 2 weeks of dietary alcohol with and without PTH treatment on bone histomorphometry. Alcohol decreased and PTH treatment increased several indices of bone formation, including LS/BS, BFR/BS, BFR/BV,

**Fig. 2.** The effects of a 1-week treatment with alcohol on steady-state mRNA levels for bone matrix proteins. Values are mean ± SE; *n* = 4. Alcohol diet (ALC), control diet (Con).

and BFR/TV. Neither treatment had a significant effect on MAR, although there was a strong tendency for alcohol to reduce this measurement. Alcohol significantly reduced but did not prevent the stimulatory effect of PTH on bone formation.

The effects of PTH treatment with and without alcohol on steady-state mRNA levels for bone matrix proteins are shown in Table 4. PTH increased mRNA levels for type 1 collagen, osteocalcin, and osteonectin. Alcohol had no significant effect on mRNA levels for the three bone matrix proteins after 2 weeks of treatment. However, it tended to decrease the mRNA levels for type 1 collagen and osteocalcin and tended to decrease the stimulatory effects of PTH on mRNA levels for all three bone matrix proteins.

DISCUSSION

Alcohol abuse leads to decreased bone formation in humans (Bikle et al., 1993; Gonzalez-Calvin et al., 1993; Labib et al., 1989; Laitinen et al., 1992; Nielsen et al., 1990; Schnitzler and Solomon, 1984) and experimental animals (Sampson, 1998; Sampson et al., 1997; Turner et al., 1987, 1988, 1991). The present studies demonstrate that the pronounced inhibitory effects of alcohol on bone formation in adult rats can be reversed by PTH, even while rats continue an alcohol intake comparable to alcoholics.

The inhibitory effect of alcohol consumption on bone formation occurred within 2 weeks of initiating treatment. The decreased mRNA levels for bone matrix proteins and strong tendency toward reduced dynamic bone measurements observed in the alcohol-consuming baseline group suggest that the inhibitory effects of alcohol on bone formation were established prior to initiation of PTH treatment. The failure to detect significant changes in dynamic bone histomorphometry in the alcohol-treated rats after 1 week is not unexpected. The fluorochrome based measurements would underestimate an inhibitory response because LS, MAR, and BFR are calculated using two fluorochrome labels, one of which was administered at the time treatment was started, at which time bone formation would not differ

Table 3. Effect of PTH on Bone Histomorphometry in Rats Fed Alcohol for 2 Weeks

Measurement	Group				Two-Way ANOVA		
	Control diet + vehicle	Alcohol diet + vehicle	Control diet + PTH	Alcohol diet + PTH	Alcohol	PTH	Interaction
BV/TV (%)	22.2 ± 1.9	29.4 ± 2.2	27.1 ± 1.9	24.9 ± 1.5	NS	NS	NS
Tb.Th (μm)	55.2 ± 2.2	60.0 ± 2.9	64.7 ± 2.4	59.8 ± 1.8	NS	NS (.06)	NS
Tb.Sp (μm)	204.6 ± 18.7	165.9 ± 12.8	182.4 ± 15.7	186.7 ± 14.8	NS	NS	NS
Tb.N (mm ⁻¹)	4.0 ± .2	4.7 ± 0.4	4.2 ± .2	4.1 ± .2	NS	NS	NS
LS/BS (%)	4.8 ± 1.3	1.0 ± .4	30.1 ± 3.1	11.7 ± 1.8	.0001	.0001	.0007
MAR (μm/d)	1.09 ± .06	.88 ± .02	1.15 ± .04	.98 ± .03	NS (.06)	NS	NS
BFR/BS (μm ³ /μm ² /d)	.05 ± .02	.01 ± .005	.35 ± .04	.12 ± .02	.0001	.0001	.0009
BFR/BV (%/d)	.19 ± .05	.04 ± .01	1.10 ± .12	.40 ± .07	.0001	.0001	.001
BFR/TV (%/d)	.04 ± .01	.01 ± .004	.29 ± .04	.08 ± .009	.0001	.0001	.0005

BV/TV, bone volume/tissue volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; LS/BS, label surface/bone surface; BFR/BS, bone formation rate/bone surface; MAR, mineral apposition rate; BFR/BV, bone formation rate/bone volume; BFR/TV, bone formation rate/tissue volume. NS, not significant.

Table 4. Effect of PTH on Steady-State mRNA Levels for Bone Matrix Proteins in Rats Fed Alcohol for 2 Weeks

Group	Type 1 Collagen/18S	Osteonectin/18S	Osteocalcin/18S
	Collagen/18S	Osteonectin/18S	Osteocalcin/18S
Control diet	10.4 ± 2.9	3.7 ± 0.9	12.0 ± 2.9
Alcohol diet	9.1 ± 1.7	3.9 ± 0.4	11.6 ± 0.7
Control diet + PTH treated	53.8 ± 4.6	16.3 ± 2.1	54.5 ± 3.6
Alcohol diet + PTH treated	35.3 ± 7.1	11.1 ± 1.2	39.1 ± 9.1

Two-way ANOVA			
Effect of Alcohol	NS (<i>p</i> = 0.05)	NS (<i>p</i> = 0.08)	NS
Effect of PTH	<i>p</i> < 0.0001	<i>p</i> < 0.0001	<i>p</i> < 0.0001
Interaction	NS (<i>p</i> = 0.09)	NS (<i>p</i> = 0.06)	NS

Values are mean ± SE; *n* = 4.

NS, not significant.

from the controls. Although the present study was only 2 weeks in duration, we have shown that bone formation is also suppressed in adult rats that were continuously fed a diet in which ethanol contributed 35% of the caloric intake after 2 (Turner, unpublished data, 2001) and 4 months (Turner et al., 2001).

The inhibitory effects of alcohol on steady-state mRNA levels for bone matrix proteins were transient; mRNA levels returned toward normal after 2 weeks of alcohol consumption. This finding was, not surprisingly, based on the results of time course studies showing that the effects of alcohol on gene expression can change dramatically over short intervals (Turner et al., 1998a), which suggests that the mRNA levels for bone matrix proteins may depend upon the interval between feeding and sacrifice.

PTH resulted in histomorphometric changes consistent with those reported in published studies, including increases in mineralizing surface and bone formation rate (Dobnig and Turner, 1995; Turner et al., 1998b). The large increases in mRNA levels for bone matrix proteins after PTH-treatment have also been reported (Dobnig and Turner, 1995). The lack of an effect of PTH on bone area and architecture was anticipated because of the short duration of the study (Dobnig and Turner, 1995; Turner et al., 1998b).

In the current study, LS was measured as an index of osteoblast number, and MAR was calculated as an index of osteoblast activity (Turner, 1994). Alcohol consumption inhibited bone formation by decreasing LS and had little

effect on MAR. These findings are in agreement with prior rat (Sampson, 1998; Sampson et al., 1997; Turner et al., 1987, 1988, 1991) and human studies (Bikle et al., 1985, 1993; Diez et al., 1994; Gonzalez-Calvin et al., 1993; Lalor et al., 1986; Pumarino et al., 1996; Schnitzler and Solomon, 1984). PTH increases bone formation in rats by increasing osteoblast number and to a lesser extent, activity (Dobnig and Turner, 1995; Turner et al., 1998b).

Prolonged exposure to high concentrations of alcohol inhibits proliferation of cultured osteosarcoma cells (Klein and Carlos, 1995; Klein et al., 1996), immortalized cells derived from the osteoblast lineage (Maran et al., 2001), and primary osteoblastic cell cultures from chick calvaria (Farley et al., 1985). Alcohol, however, had little effect on differentiated osteoblasts which suggests that the reduction in LS in rats that consume alcohol may be due in part to decreased cell proliferation (Klein and Carlos, 1995; Klein et al., 1996; Maran et al., 2001). On the other hand, modulation of osteoblasts to the quiescent bone lining cell phenotype would also result in a reduction in LS. This latter explanation is more likely because the rate of osteoblast turnover in adult rats is very slow (Dobnig and Turner, 1995).

In the present study, chronic alcohol consumption blunted the anabolic effects of PTH on bone. Others have reported that alcohol reduces PTH levels in humans (Laitinen et al., 1991b) and antagonizes the hypercalcemic effects of PTH in rats and dogs (Peng and Gitelman, 1974; Peng et al., 1972). Thus, reduced circulating levels of PTH and/or end organ resistance to the hormone may contribute to alcohol-induced bone loss. Despite the observed antagonism, PTH-treatment increased bone formation in the alcohol-treated rats to values which greatly exceeded those observed in rats fed the control diet. Furthermore, PTH treatment reversed the reduction in LS observed after chronic alcohol consumption. These findings suggest that PTH treatment may be useful as an intervention to reverse alcohol-induced osteopenia. However, additional long-term studies will be necessary to demonstrate that PTH treatment is capable of restoring bone mass in rats with alcohol-induced osteopenia.

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EFFECTS OF ALCOHOL AND ESTROGEN
ON BONE METABOLISM

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Abstract

Not much direct attention has been placed upon the differential skeletal response of women vs. of men to alcohol. In marked contrast to men, women who drink are often found to have more bone than women who abstain do. Furthermore, the apparent beneficial effects of drinking are more apparent in postmenopausal women, suggesting that there might be an interaction between alcohol and estrogen signaling. Estrogen deficiency accompanying the menopause leads to bone loss, which in turn predisposes these women to developing osteoporosis later in life. Estrogen deficiency accelerates bone remodeling and leads to an imbalance whereby bone resorption exceeds bone formation. Alcohol might reduce bone loss in postmenopausal women by increasing the circulating levels of estrogen. Alternatively, alcohol might slow bone loss by acting on bone cells to reduce bone remodeling. Our current understanding suggests that alcohol has a negative effect on the growing bone but that small quantities of alcohol may have beneficial effects on the skeleton in older women.

Key Words: postmenopausal osteoporosis; alcoholism; moderate drinking; calcium homeostasis; fracture risk

Introduction

Bone is a living tissue which undergoes life long remodeling (Frost, 1969). Remodeling is a process whereby local regions of the bone are destroyed and rebuilt in a systematic way (Figure 1). This process serves to repair microdamage caused by fatigue and is essential to maintain strong bones. Unfortunately, bone is lost during the normal aging process.

Osteoporosis occurs when there is an inadequate amount of bone to provide sufficient strength to perform normal daily activities. Osteoporosis is usually caused by a chronic imbalance in the bone remodeling cycle in which bone resorption (Figure 1A and 1B) is not adequately compensated for by subsequent bone formation (Figure 1C and Figure 1D).

Bone mass is not constant; it reaches a peak value in young adults and then declines with age. A low peak bone mass and a rapid age-related loss in bone mass predispose an individual to development of osteoporosis. The onset of bone loss during middle age can precede an increased risk of fractures, by as much as two decades, during which period the individual is asymptomatic. Anabolic agents are under investigation but at present there is no method to restore small amounts of bone to a depleted skeleton. As a consequence, reducing the rate of bone loss is the only effective way to delay osteoporotic fractures. Two important controllable risk factors for osteoporosis are estrogen deficiency and alcohol abuse. This article will highlight these two factors by discussing their respective effects on bone metabolism and the possible influence of alcohol on the skeleton of estrogen-deficient women.

Medical and Public Health Costs of Musculoskeletal Disorders

The true extent of musculoskeletal disorders in the general population is not widely appreciated. Musculoskeletal complaints are among the most common reasons why patients see physicians and are hospitalized. The annual direct and indirect costs of musculoskeletal

disorders in the United States exceed \$125 billion. Direct treatment amounts to a little less than half of the economic burden; morbidity, mortality, and the value of lost productivity account for most of the remaining costs.

There are over 6 million bone fractures in the United States per year of which roughly 5% do not heal properly and require additional and often costly medical care. Only 1/6th of the fracture total is due to osteoporosis. However, the consequences of osteoporotic fractures can be disproportionately severe. Hip fractures are an especially dangerous fracture with more than 275,000 occurring annually in the U.S., 2/3rds of which are due to osteoporosis. The common perception that a fracture is not life threatening is wrong; one in five patients with a hip fracture dies within 6 months and those who survive generally have a poor prognosis for complete recovery. Of the survivors, 1/5th will require long institutionalization and another 1/5th face a permanent disability, often with mobility limited to a walker or wheelchair (Cummings, 1985). The prevalence of osteoporosis and the number of hip fractures are increasing at an alarming rate. The second half of the last century has witnessed an unprecedented increase in lifespan. Longer life combined with the transient increase in birth rate in the years immediately following the Second World War will greatly increase the number of Americans at risk for osteoporosis in the next two decades.

Estrogen Deficiency as a Risk Factor for Osteoporosis

Estrogens influence virtually all aspects of bone physiology throughout life (Turner, 1993). The hormone plays an important role in maintaining bone mass in the adult woman, in part by slowing bone remodeling and in part by maintaining the proper balance between osteoblastic (bone forming cells) and osteoclastic (bone resorbing cells) activity. The effects of estrogen and estrogen deficiency on bone metabolism are summarized in the Table. When

estrogen is deficient, there is an increase in the activation of new bone remodeling units. A remodeling unit is the discrete region on a bone surface where the remodeling sequence shown in Figure 1 occurs. Increased activation frequency can result in an increase in fracture risk. This is because the resorption and formation phases of the bone remodeling cycle are separated in time. As shown in Figure 1A and B, resorption of bone by osteoclasts creates a cavity on the bone surface. This local thinning creates a "weak link" in the trabecular network consisting of fine spicules of bone that could cause it to catastrophically fail under load. The greater the remodeling rate, the greater the number of "weak links."

Added to this immediate degradation of bone strength, is the additional effect of estrogen deficiency on bone remodeling balance. Estrogen reduces osteoclast lifespan and lengthens the lifespan of the bone forming cells. In estrogen deficient women, osteoclasts are believed to excavate deeper resorption cavities which osteoblasts are unable to completely refill. This leads to a negative remodeling balance in which there is a small amount of bone lost at every location where bone has undergone remodeling. The combination of increased bone remodeling units and a negative remodeling balance are the basis of the rapid decrease in bone mass which follows menopause.

Once bone mass has declined below a critical threshold, fractures may occur after minimal or no trauma. When bone mass falls to this level, with or without an existing fracture, the patient is diagnosed as having osteoporosis. This critical threshold is approximately at the lower limit of the normal range for premenopausal women. As bone loss continues, the risk for fractures increase. The spongy appearing cancellous bone located in the marrow cavity and more compact cortical bone forming the shaft is at risk. Over a lifetime, women lose about 50% of their peak cancellous bone and about 35% of their peak cortical bone. It is believed that the

excessive bone loss due to estrogen deficiency is the most important of the many factors that determine the overall risk for osteoporosis in women (Richelson, 1984).

Menopause occurs in all women as they progress through middle age. Osteoporosis, however, is not inevitable. Thus, it is clear that other factors influence this process. Genetics is important, of course. In general, African Americans are at a lower risk for osteoporosis than Americans of European ancestry. In addition, life style choices play an important role in maintaining skeletal health.

Alcohol as a Risk Factor for Osteoporosis

There is overwhelming evidence from human and laboratory animal studies that chronic alcohol abuse has detrimental effects on the skeleton (reviewed in Turner, 2000). The development of osteoporosis in middle aged men is uncommon in the general population but is often associated with alcoholism. Decreases in bone mass in alcoholics have been documented by radiography, densitometry, and histology. Histological studies have shown that alcoholics have reductions in the total amount of cancellous bone as well as in the thickness of individual bone spicules. There is evidence, primarily from laboratory animals, that alcohol also results in the production of bone with sub-optimal mechanical properties (Hogan, 1997). As a consequence, bone strength in alcoholics might be even less than predicted from bone mass measurements. The effects of alcohol on bone metabolism during childhood and adolescence have not been studied but extensive studies have been conducted in growing rats (Sampson, 1997). Extrapolation from laboratory animal data strongly suggests that underage drinking results in an immediate increase in fracture risk and, in addition, predisposes the skeleton to osteoporotic fractures later in life.

Gender Differences in the Skeletal Response to Alcohol

The great majority of studies to assess the effects of alcohol on bone and mineral homeostasis have been performed in men. Because of the limited number of studies, the magnitude of risk posed by alcohol has not been reliably established for women. In contrast to men, however, there is little evidence for bone loss in female alcohol abusers. Indeed, alcohol abuse was associated with an increased fracture rate in men but not in women (Reviewed in Turner, 2000).

The skeletal effects of moderate alcohol consumption on bone and mineral homeostasis are relevant to a greater number of people but are even less certain. Reports of improved bone mass, especially among postmenopausal women, are intriguing (Laitinen, 1993). However, additional studies in rats that indicate a suppression of bone formation with alcohol consumption as low as 3% of caloric dose (Turner 2000) emphasize the need for more definitive studies

Action of Alcohol on Bone Growth and Remodeling

The effects of alcohol on bone metabolism are summarized in the Table. Histological studies in alcoholics have shown that alcohol abuse is associated with decreased osteoblast activity (reviewed by Turner, 2000). The histological evidence for disturbed bone remodeling is supported by consistent findings of reduced serum osteocalcin, a biochemical marker of bone formation. In contrast, the reported effects of alcohol abuse on histological and biochemical markers of bone resorption show no consistent change. Dose response studies in adult rats have shown that moderate alcohol consumption (defined as alcohol contributing 3-6% of caloric intake) decreased bone formation and bone resorption whereas abusive levels of alcohol (>10% of caloric intake) resulted in further decreases in bone formation with no additional decrease in bone resorption. As a consequence, bone loss occurred at these high dose rates (Turner, 2001).

Moderate as well as abusive alcohol consumption reduces bone remodeling in rats. Although the dose response effects of ethanol on the human skeleton are not known, there is evidence that only small amounts of alcohol are required to reduce serum osteocalcin, suggesting that alcohol results in a similar decrease in bone remodeling in humans (Nielsen, 1990).

The observed changes in osteoclast and osteoblast numbers suggest that alcohol may reduce the rate of initiation of bone remodeling sites at all consumption levels. In addition, abusive levels of alcohol may antagonize coupling between bone formation and bone resorption. One possible mechanism for these actions is the known interference by alcohol in the local expressions of the cytokines that mediate bone remodeling and the coupling of bone formation to bone resorption. In support of this hypothesis, the expression of insulin-like growth factor-I, an important survival factor for osteoblasts in bone, has been shown to be reduced by alcohol (Turner, 1998).

On the other hand, not all alcoholics exhibit a low bone mass. Furthermore, it has been difficult to demonstrate either alcohol-induced bone loss or increased fracture rate in population-based studies. Indeed, most studies have shown a positive association between alcohol and bone mass and no change or a decrease in fracture risk. Overall, the evidence generally supports a detrimental effect of chronic alcohol abuse on the skeleton of a sub-population of men and a neutral or generally beneficial skeletal effect for moderate alcohol consumption, especially in older women. This variability in the skeletal response to alcohol strongly implicates an important role for co-risk factors.

The apparent contradictory effects of alcohol on bone mass in young men and women compared to postmenopausal women may be reconciled by considering the effects of drinking on bone growth and remodeling. The anticipated inhibitory effects of alcohol on bone growth in

adolescent boys and girls would lead to a reduction in peak bone mass which would in turn predispose those individuals to bone fractures later in life. An imbalance between bone formation and resorption in young adult male and female alcohol abusers, particularly when the latter predominates, would lead to gradual bone loss despite decreased bone remodeling. In contrast, a decrease in bone remodeling in older women who consume alcohol would slow bone loss relative to their peers despite having a negative remodeling balance.

Action of Estrogen on Bone Remodeling

Estrogen's influence on the growth process of long bones, cancellous bone density, as well as on the architectural and cellular changes in bone has been described in greatest detail in laboratory animals. Ovariectomy results in severe cancellous osteopenia in long bones and vertebrae of rats (Wronski, 1989) and in the vertebrae of monkeys (Jerome, 1986). In the long bones of rats, ovariectomy results in increases in bone surfaces lined by osteoblasts and osteoclasts. There are concurrent increases in the mineral apposition and bone formation rates, suggesting that ovariectomy results in increased remodeling activation frequency. Ovariectomy of skeletally mature female rats resembles menopause in that cancellous and cortical bone loss occur due to an abnormally high rate of bone remodeling superimposed on a negative remodeling balance. The elevated cancellous bone turnover persists in rats a year or more after ovariectomy. In the ovariectomized monkey bone formation is increased, suggesting that bone loss in non-human primates is also associated with increased bone remodeling.

Replacement of estrogen stabilizes cancellous bone volume in ovariectomized rats by reducing the rate of activation of new bone remodeling and re-establishing a neutral or positive balance between bone formation and bone resorption during the remodeling cycle. Estrogen replacement in postmenopausal women was also reported to result in a positive remodeling

balance because the volume of bone removed during the resorptive phase of the remodeling cycle is exceeded by the volume of new bone deposited during the formation phase (Figure 1D). Thus, there seems to be overwhelming evidence that 17β -estradiol reduces the overall rate of bone remodeling and improves remodeling balance in humans and animals compared to estrogen deficiency.

In women, gonadal insufficiency after the menopause greatly accelerates bone remodeling with a net increase in bone resorption. Inhibitors of bone remodeling, which limit the initiation of new remodeling sites, universally reduce the rate of bone loss as well as reduce fracture risk. An inhibitory effect of moderate alcohol on bone remodeling would be consistent with studies that report higher bone mass in postmenopausal women who drink alcohol. It should be emphasized that this difference in bone mass represents a relative improvement; it is unlikely that these women are gaining bone. It is more likely that they are losing bone more slowly than their peers are.

Interaction between Alcohol and Estrogen Signaling

In addition to the described independent effects of alcohol and estrogen on bone metabolism there is evidence that alcohol influences estrogen signaling. Chronic male alcoholics develop an assortment of endocrine disorders, including infertility, gonadal atrophy, hypoandrogenization and feminization, due in part to elevated production of estrogens and low serum testosterone. Testosterone is converted to estrogen in peripheral tissues including bone by aromatase, and the activity of this enzyme is increased by alcohol (Chung, 1990). It is also possible that the higher bone mass reported in women who drink alcohol is due to higher circulating levels of estrogen.

The acute and chronic effects of alcohol on serum and urine estrogens have been investigated with mixed results (Purohit, 1998). Interestingly blood levels of estrogen were higher in hormone-replaced postmenopausal women who drank alcohol and than in those who abstained. Increased aromatase activity cannot explain this finding. Instead, it suggests that alcohol slowed the metabolism of estrogen in these women.

Laboratory animal studies have shown that alcohol can increase the number of receptors for estrogen in the liver (Chung, 1990) and may account for the increased transcriptional activity of ligand-bound estrogen receptor in human breast cancer cell lines (Fan, 2000). If similar changes in receptor number were to occur in bone cells, alcohol might increase the sensitivity of the skeleton to circulating levels of estrogen. However, alcohol has no consistent effect on the uterus in ovariectomized rats and tends to decrease uterine weight in ovary-intact rats. The failure to detect a stimulation of uterine weight argues against an increase in sensitivity to normal circulating levels of estrogen.

Summary

Based on limited evidence, the skeleton of women appears to respond differently to alcohol than that of men. In general, the pronounced detrimental effect of alcohol abuse to decrease bone mass and thereby increase fracture risk is less prevalent in females than in males. Indeed, the weight of evidence suggests that women who consume alcohol generally have a higher bone mass than nondrinkers do. There are at a minimum two plausible ways that alcohol could bring about these putative beneficial effects on the skeleton. Alcohol could enhance estrogen signaling by increasing the circulating levels of the hormone or by increasing the number of estrogen receptors in bone cells. Alternatively, direct effects of estrogen on bone cells

to inhibit initiation of bone remodeling would have beneficial skeletal effects in older women.

These alternative actions of alcohol need not be mutually exclusive and may be additive.

As with the skeletal effects of estrogen, any beneficial effects of consuming alcohol are context dependent and should not be viewed in isolation. Whereas estrogen deficiency-induced bone loss leads to fractures in some women which can be prevented by hormone replacement therapy, estrogen replacement has been implicated as a positive risk factor for breast cancer, another life threatening disease. The evidence strongly supports the conclusion that alcohol is detrimental to the growing skeleton. Furthermore, the many detrimental effects of alcohol abuse on other organ systems contraindicate any beneficial effect of heavy drinking on the female skeleton. On the other hand, because of the importance of osteoporosis as a life threatening and debilitating disease with no known cure, even a modest reduction in bone loss could have important positive public health implications. Further, research needs to determine if and under what circumstances moderate alcohol consumption may offer some protection against bone loss in aging women.

HOW ALCOHOL MIGHT INFLUENCE BONE MASS IN ADOLESCENT,
ADULT AND POSTMENOPAUSAL WOMEN

Response	Gonadal Status	Alcohol
Adolescent	Estrogen	Estrogen & Alcohol
Growth	↓	↓
Density	↑↑	⇔
Young Adult	Estrogen	Estrogen & Alcohol
Remodeling Rate	↓	↓
Remodeling Balance	⇔	↓
- Formation	↓	↓
- Resorption	↓	↓
Density	⇔	↓
Postmenopausal	Estrogen Deficiency	Alcohol
Remodeling Rate	↑↑	⇔
Remodeling Balance	↓	↓
Formation	↑↑	⇔
Resorption	↑↑	↑↑
Density	↓	↓
Size of arrow represents the magnitude of change; ↑↑ indicates increase; ↓↓ indicates decrease; ⇔ indicates no net change.		

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Figure Legend

Figure 1: The bone remodeling cycle.

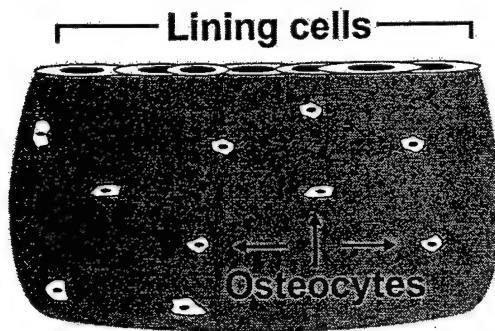
(A) Quiescent phase - Inactive bone with surface lined with bone lining cells. Neither bone resorption nor formation is occurring on this region of bone surface.

(B) Resorption phase - Osteoclast-mediated bone resorption. The osteoclasts remove a discrete packet of bone, creating a lacunae.

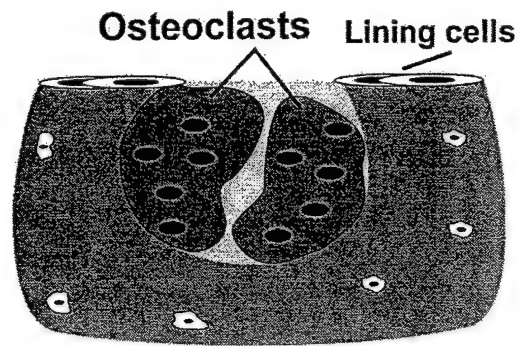
(C) Formation phase - Osteoblasts form bone matrix which falls in the lacunae. The cement line defines the boundary between the newly formed bone and the surface of the lacunae excavated by the osteoclasts at the end of the resorption phase.

(D) Quiescent phase - Inactive bone surface that shows the completed remodeling cycle. The new surface may be underfilled (a), exactly filled (b), or overfilled (c), the resorption lacunae reflecting a local decrease, no change or increase in bone mass, respectively. The most likely mechanism for alcohol-induced bone loss in adults is underfilling of the resorption lacunae during bone remodeling.

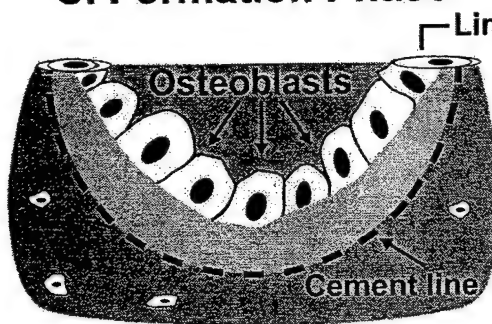
A. Quiescent Bone Surface



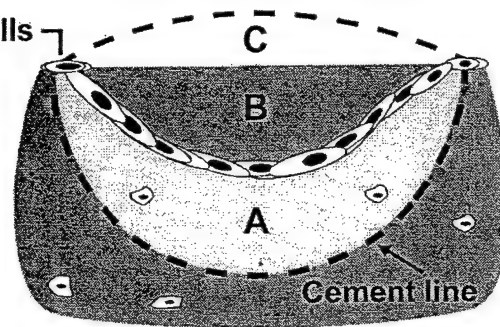
B. Resorption Phase



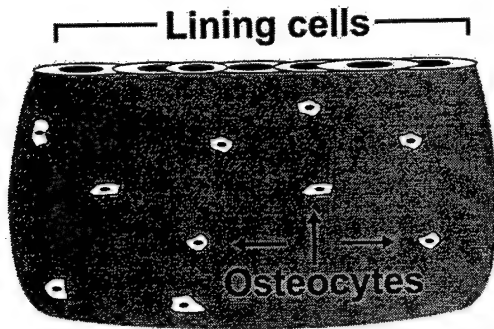
C. Formation Phase



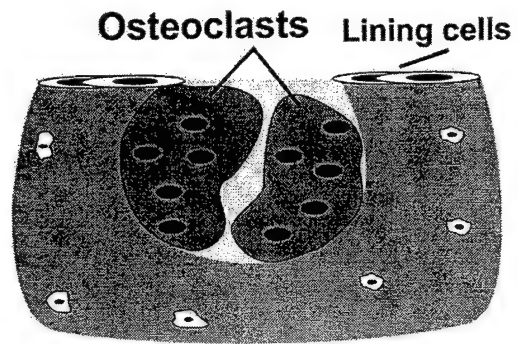
D. Quiescent Phase



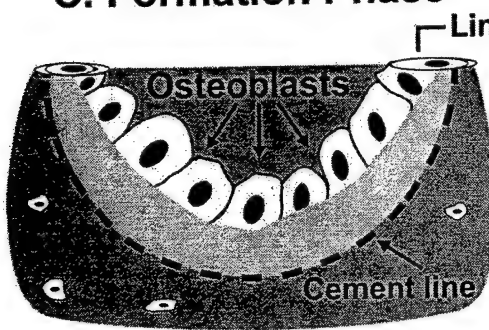
A. Quiescent Bone Surface



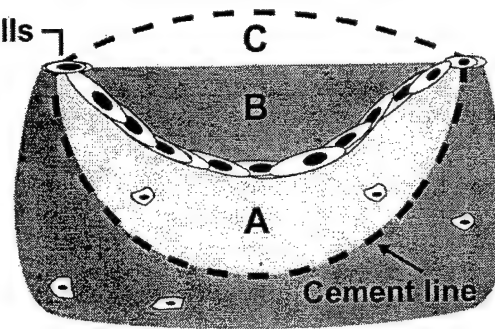
B. Resorption Phase



C. Formation Phase



D. Quiescent Phase



Author Proof

Skeletal adaptation to external loads to optimize mechanical properties: fact or fiction

Russell T. Turner, PhD

The skeleton adapts to a changing mechanical environment but the widely held concept that bone cells are programmed to respond to local mechanical loads to produce an optimal mechanical structure is not consistent with the high frequency of bone fractures. Instead, the author suggests that other important functions of bone compete with mechanical adaptation to determine structure. As a consequence of competing demands, bone architecture never achieves an optimal mechanical structure. Curr Opin Orthop 2001; 0:000-000

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C.

There is irrefutable evidence that bone adapts to changes in external mechanical loads [1-11] (Fig. 1). Three fundamental rules that govern bone adaptation to changing mechanical environments have been described by Turner [1]: 1) adaptation is driven by dynamic, rather than static, loading; 2) only a brief duration of loading is required to initiate an adaptive response; and 3) bone cells adapt to a customary mechanical loading environment, making them less responsive to similar or repetitive loading signals. *proposed*

According to these three principals, bone should adapt on the local level to form and maintain an optimal mechanical structure [4]. An obvious extension of this attractive hypothesis is the prediction that bone fractures should be uncommon. Because that is not the case, there must be a flaw in the mechanical adaptation hypothesis. It is the thesis of the author that adaptation to external loads is often superseded by other critical demands on the skeleton; as a consequence, bone architecture never achieves an optimal mechanical structure. This review considers some of the factors that compete with mechanical usage in determining bone architecture.

Effects of spaceflight on bone and mineral metabolism

Disuse caused by a variety of conditions including traumatic spinal cord injury and stroke [6,7,9-11] as well as physical inactivity secondary to renal failure [2] result in rapid bone loss and a greatly increased risk for bone fractures. It is not entirely clear as to the respective contributions of reduced mechanical usage and injury and/or disease process to the bone changes. In contrast to these illnesses, astronauts are physically fit and have no underlying disease or injury. As a consequence, the abnormalities observed in bone and mineral homeostasis during spaceflight are generally attributed to the reduced skeletal loading. Alterations in bone and mineral metabolism resulting in bone loss have been documented in astronauts during spaceflight, despite exercise, indicating that every day gravitational loading of the skeleton, which is difficult to mimic during spaceflight, plays an important role in maintaining normal bone mass and architecture. *to what degree*
This raises
and raising the concern that long-duration missions required for interplanetary exploration would have an unacceptable detrimental effect on astronaut health. The cellular and molecular mechanisms leading to, as well as countermeasures to prevent, spaceflight-induced osteoporosis are now being aggressively investigated using earth-based models for spaceflight. *1*

mechanical loading stimulates formation during bone remodeling

Figure 1. Formation During Bone Remodeling

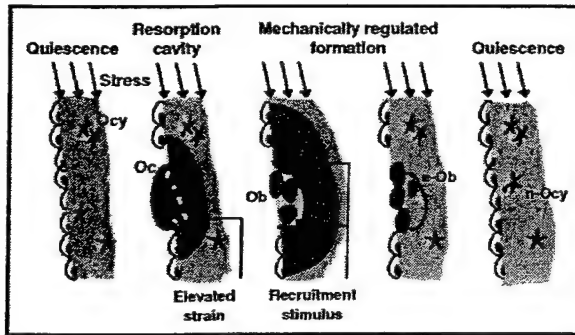


FIGURE 1 The trabecular surface is covered by lining cells (LC) and the osteocytes (Ocy) are inside the mineralized tissue. When an osteoclast (Oc) is recruited to resorb bone, a cavity is made, which weakens the trabecula and causes a local elevation of strain. After the osteoclast has gone, osteoblasts (Ob) are thought to be recruited by osteocytes to form bone. During the bone formation process, some of the osteoblasts are entrapped in the bone matrix (e-Ob), where they differentiate to new osteocytes (n-Ocy). After repair, the remaining osteoblasts become lining cells, covering the new bone surface. Published with permission [4].

Relatively few studies have been performed in astronauts during spaceflight and these have been recently reviewed [12,13,14]. Bone loss is frequently observed after spaceflight but there are large individual and site variations in the magnitude of the loss. The greatest bone losses have been observed in weight-bearing locations, including the lower body, specifically in pelvic bones, lumbar vertebrae, and the femoral neck. However, the most consistent finding to date in astronauts is that spaceflight results in chronic disturbances in mineral balance. For example, a negative calcium balance occurs

almost immediately, resulting because of a reduction of intestinal absorption and concurrent increases in excretion through the gastrointestinal tract and kidneys. This finding implies the involvement of other organ systems suggesting, in contrast with the prevailing view, that adaptation of bone architecture to major changes in external mechanical loading, is not strictly a local phenomenon. If this interpretation is correct, there is no basis to assume that the effects of altering the mechanical environment on bone tissue will automatically lead to compensatory changes in intestine and kidney that may be required for skeletal adaptation.

Mechanisms for the variations in bone loss during spaceflight are not completely understood. Measurements of biochemical markers of bone turnover are available for only a small number of astronauts. There are no consistent changes in bone formation markers; they are reported to increase, remain constant, and decrease after spaceflight. Bone resorption markers, on the other hand, are unchanged or increased after flight. Thus, it is not clear whether the bone loss that occurs in most astronauts is associated with increased bone remodeling, re-

duced bone remodeling, or an uncoupling between bone formation and bone resorption.

The skeletal adaptation to mechanical usage requires the participation of cells. The osteocyte is the leading candidate for the cell that detects mechanical signals [8,15]. Osteoblasts and osteoclasts are the cells that affect changes in bone mass. A change in mechanical loading should result in a consistent cellular response if the adaptation occurs at the local level and is due entirely to the local loading situation. However, male rats lose bone during spaceflight largely because of depressed bone formation, whereas, ovariectomized female rats lose bone because of increased bone resorption. These radically different cellular mechanisms in response to similar external loading conditions further suggest that skeletal adaptation to mechanical loads is influenced by additional systemic factors [16].

Spaceflight studies on astronauts have been largely observational. The primary goals of these missions have been varied; as a consequence they have differed greatly in duration as well as in the physical demands placed on the crew. Diet and activity levels typically vary between missions as well as between individual astronauts. Ground-based laboratory studies in humans and ground-based and spaceflight studies in laboratory animals can be more easily controlled and have yielded the bulk of our knowledge concerning the skeletal response to changes in gravitational loading [12]. In contrast to astronauts, animals are not subjected to an exercise program. There is limited evidence in rats that the non-weight-bearing bones are less affected by spaceflight than weight-bearing bones and that the skeletal effects of spaceflight are progressive. However, changes are not always detected in weight-bearing bones after spaceflight. These negative studies suggest that weightlessness does not always result in bone loss. The effects of spaceflight may be influenced by housing conditions, age, or other, as yet unknown factors which need to be identified and characterized before we can be certain as to how to best minimize the detrimental effects of space travel on astronauts. An important take-home message from these studies is that dramatic changes in mechanical usage do not necessarily lead to skeletal adaptation.

Structure to function relationships in the skeleton

It has long been recognized that structure and function are intimately related in biology and that the skeleton is no exception. Structure to function relationships in the skeleton cannot be precisely calculated using our current understanding of physical laws, probably because they are largely the result of trial and error processes driven by evolutionary pressures that defy computational analysis. There have, however, been significant advances [4•]. Attempts to describe skeletal development, repair, and

remodeling based on mechanical considerations alone [2] are unlikely to be completely satisfactory. This is partly because a skeleton is created by the activities of living cells whose proliferation, differentiation, and activity are subject to many biologically based constraints. These biologic constraints may decrease the ability of the adult skeleton to respond to vigorous exercise [17]. Furthermore, optimization of the mechanical function of the skeleton competes with its other functions, each of which may require a different structure for optimal performance.

At first glance, the overriding function of the skeleton is to resist applied mechanical forces. And in point of fact, the skeleton fulfills several essential mechanical functions such as providing a scaffold for the attachment of muscles, ligaments, and tendons necessary for locomotion, and as a barrier to protect vital organs. Not surprisingly, applied mechanical force is an important determinant of bone mass and architecture. However, the critical need to maintain blood ionic calcium levels within narrow parameters on a moment-to-moment basis can cause changes in bone metabolism, which over the long-run can compromise the mechanical function of bone.

The knowledge that complete disuse leads to rapid bone loss whereas exercise has minimal effects on bone mass in healthy active individuals is evidence for the hypothesis that there is a sophisticated mechanical sensing system in bone, sometimes referred to as the "mechanostat" [1,40,17]. On the other hand, more than six million fractures occur each year in the United States. These seemingly contradictory facts could be easily reconciled if the bone fractures were entirely caused by ^{either} extreme trauma or ^{alternatively} to diseases that compromise the ability of bone cells to respond to mechanical signaling. However, many fractures do not fit either of these criteria. Another possibility is that age-related bone loss occurs in spite of the mechanical sensing system because of an inherent defect in the ability of the aging skeleton to form bone. This possibility was recently tested in animals [18]. Rats were ovariectomized at a young age to model gonadal insufficiency. Treatment was delayed for 20 months by which time the animals developed severe osteopenia. These senescent rats were then treated for two months with PGE₂, a potent stimulator of *de novo* bone formation. Treatment dramatically increased bone mass to values that did not differ from normal, indicating that aging does not necessarily diminish the capacity of the skeleton to form bone. These findings lead to the conclusion that mechanical signaling has a very limited capacity to affect skeletal adaptation ^{in the absence of normal circulating levels of gonadal hormones.}

Material but not mechanical properties of bone have been optimized for strength

- * The material properties of bone, which have been highly conserved during evolution, ~~depend upon the composition~~

* The material properties of bone are determined by the composition of the organic and mineral components whereas the mechanical properties are determined by the material properties and architecture.

tion of the organic and mineral components. The mechanical properties of bone depend upon the material properties and architecture. A single amino acid substitution in type I collagen is responsible for striking ^{deterioration} ~~dramatic~~ changes in bone material properties that characterize osteogenesis imperfecta. In contrast, ^{to the similarity} ~~with the~~ organic and mineral composition, there are dramatic species differences in bone architecture, that play an important role in determining bone mechanical properties. Interesting studies have been performed comparing the responsiveness of high bone mass and low bone mass mouse strains to mechanical loading. There were large differences, with the low bone mass mice found to be much more responsive [19,20]. This finding clearly demonstrates the capacity of natural selection to neutralize mechanical loading as an important factor in controlling bone architecture. Thus, it is likely that material but not mechanical properties of bone have been optimized for strength.

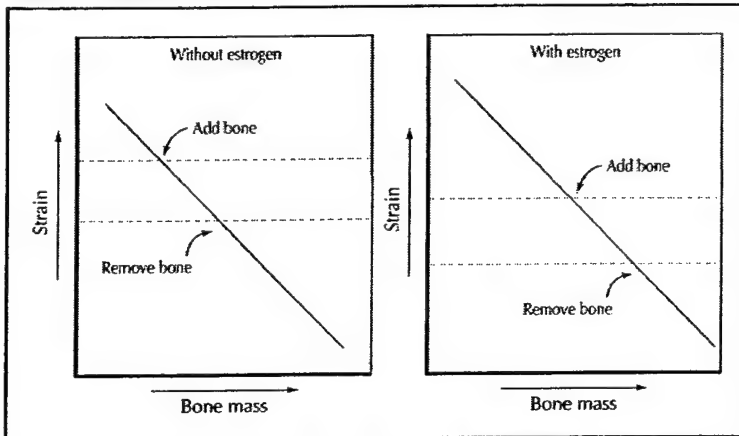
Reproduction—an important determinant of bone architecture

The near extinction of several species of raptors in the 20th century, including the bald eagle, golden eagle, and peregrine falcon, as well as the near catastrophic decline of the brown pelican, was caused by a defect in the transport of calcium from the skeleton to the egg shell due to exposure to the insecticide DDT. As a result, the shell contained inadequate mineral to provide sufficient mechanical strength to support the weight of the parent during incubation. DDT is a weak estrogen and is thought to have acted as an endocrine disrupter.

The uncompromising requirement of migratory flight for skeletal strength and lightness has resulted in the nearly complete separation of the mechanical and reproductive functions of bone in birds. After migration to the nesting site and before the onset of egg laying female birds grow a novel form of bone *de novo* in the marrow cavity under the influence of sex hormones, primarily estrogen, called medullary bone. Mineral is mobilized from this bone during egg laying and the bone completely disappears after reproduction and before winter migration. Although superficially similar in structure to the normal weight bearing cancellous bone, medullary bone has no mechanical function.

Although the role of the skeleton in reproduction can be most vividly illustrated in birds, there is no doubt that bone is critically important for reproduction in mammals as well. Increasing estrogen levels during adolescence in women leads to increased bone volume over and above that required to satisfy mechanical requirements [21]. This excess bone ^{is} ~~can be~~ mobilized during pregnancy and lactation. However, to store excess bone, the putative mechanical sensing system would have to be altered so that bone subjected to less than optimal strain would

Figure 2. Proposed relation between strain and bone mass in presence and absence of estrogen



The horizontal lines represent the upper and lower set points for mechanical adaptation. Estrogen lowers the set points such that bone is added at lower strains. As a consequence, higher bone masses are maintained in the presence of estrogen. Published with permission [24].

be preserved by adjusting the sensitivity of the mechanostat to mechanical loading (Fig. 2). This hypothesis could account for the rapid bone loss that follows menopause; the decrease in estrogen levels would be expected to result in loss of this protected bone.

Ovariectomized adult rats lose cortical bone and cancellous bone in a manner that is very similar, if not identical, to postmenopausal women. As in the case of women, the bone loss is not uniform. Finite element modeling has revealed that bone is lost from sites that experience the lowest mechanical strain energy, further suggesting that there is an intimate relation between the effects of estrogen and mechanical usage on bone integrity [22]. The interaction of mechanical usage and estrogen has been investigated during orbital spaceflight. Rats lose more bone in space than can be attributed to estrogen deficiency alone. Ovariectomized rats in orbital spaceflight also lose bone from skeletal sites that are normally preserved in mechanically loaded animals and ovary-intact unloaded rats. On the other hand, increased mechanical loading caused by treadmill exercise reduces bone loss in ovariectomized rats. These findings in laboratory animals strongly support the idea that estrogen deficiency-induced bone loss is caused by a defect in the mechanosensory system that can be corrected by hormone replacement therapy. A theoretical model was developed to predict cancellous bone structure after changes in mechanical loading and estrogen. The model was validated experimentally by simulation of the normal structure of a rat distal femur, and further used to predict the structural alterations caused by ovarian hormone deficiency. Thus, by including other factors, it is possible to simulate the changes in bone mechanical properties that contribute to fracture risk [23].

An important reproductive function for the female skeleton seems certain, but what about males? The respec-

tive privileges and responsibilities of males and females in mating rituals show pronounced cultural differences. However, physical appearance plays an important role in the process of mate selection and the skeleton is one important determinant of physical appearance. Physical characteristics leading to less than optimal bone biomechanical properties but a greater likelihood of successful reproduction can become dominant as a result of natural selection.

Summary

In summary, the weight of evidence supports the view that mechanical usage is one of numerous factors that compete with one another to determine bone architecture. A more complete understanding of their respective contributions will better enable the development of computational models to predict bone structure, which in turn increases the likelihood that it will be possible in the future, using tissue engineering and other techniques, to restore bone architecture in individuals who are at risk for fractures, because of a deficiency in either bone mass or architecture, and/or bone mass can be restored.

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AU2

Mechanical signaling in the development of postmenopausal osteoporosis.

Triazolopyrimidine (trapidil) inhibits the detrimental effects of parathyroid hormone in
an animal model for chronic hyperparathyroidism

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Running Title: PDGF-A induced marrow fibrosis

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marrow fibrosis

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The skeletal disorder osteitis fibrosa cystica, characterized by peritrabecular fibrosis and increased bone resorption, is caused by chronic hyperparathyroidism and is a common feature of renal osteodystrophy. Using a cDNA microarray having over 5000 genes, we identify platelet-derived growth factor-A (PDGF-A) as a putative causative factor for parathyroid hormone (PTH)-induced bone marrow fibrosis in rats. Verification of PDGF-A over-expression was accomplished by an RNase Protection Assay. We also report a novel strategy for the treatment of osteitis fibrosa cystica using triazolopyrimidine (trapidil), a PDGF antagonist. Trapidil itself did not have any effect on bone histomorphometry values. However, a dramatic reduction in marrow fibrosis and osteoclastic resorption was observed in PTH-treated rats given trapidil. These results suggest that PDGF signaling is essential for the detrimental effects of PTH excess, and drugs which target the cytokine or its receptor might be useful in reducing or preventing skeletal pathology occurring in primary and secondary hyperparathyroid patients.

Parathyroid hormone (PTH) is a major physiological regulator of bone metabolism. However, chronic elevation of PTH level in humans leads to a metabolic bone disease known as osteitis fibrosis cystica^{1,2}. This skeletal disorder is now rarely encountered in primary hyperparathyroidism because of early intervention but frequently occurs in poorly managed renal osteodystrophy³. Renal osteodystrophy occurs in patients with chronic renal failure and in essence is a disorder of bone remodeling. A renal impairment in the conversion of 25-hydroxyvitamin D₃ to 1 α , 25-dihydroxyvitamin D₃ and the excretion of phosphate results in hypocalcemia and phosphate retention, leading to a chronic increase in PTH secretion^{4,5}. The stage of skeletal changes in chronic hyperparathyroidism depends on the severity and duration of the disease: (i) dissecting osteitis, tunneling of trabeculae by osteoclasts and an excess of osteoid formation by osteoblasts; (ii) osteitis fibrosa (Fig. 1*a*), bone resorption accompanied by fibrosis around the weakened trabeculae; (iii) osteitis fibrosa cystica (Fig. 1*b*), replacement of marrow by fibrous tissue, hemorrhage from microfractures and hemosiderin-laden macrophages that often display multinucleated osteoclast-like giant cells resulting in a cystic brown tumor⁶. The etiology of these histological changes is incompletely understood. Treatments currently used to control this skeletal disease are vitamin D supplementation and partial parathyroidectomy, which reduce symptoms but can lead to undesirable side effects, including adynamic bone disease.

To find therapeutic alternatives to PTH-induced bone damage, an animal model for osteitis fibrosa cystica was developed. Continuous infusion of PTH into rats via a subcutaneous implanted osmotic pump for 6 days results in skeletal changes that are similar to abnormalities observed in patients with chronic hyperparathyroidism, including

peritrabecular marrow fibrosis and focal bone resorption. In contrast, daily s.c. injection of PTH induces bone formation without detrimental skeletal effects⁷. The histological presentation of the skeletal abnormalities suggests that continuous PTH results in release of growth factors by osteoblasts which are chemotactic and mitogenic to fibroblasts. To test this hypothesis in rats, we used a cDNA microarray to identify candidate genes which mediate the skeletal changes and to develop a therapeutic strategy for osteitis fibrosa cystica.

Continuous PTH induces osteitis fibrosa

Using an *in vivo* rat model, we confirmed the effect of two types of PTH administration on bone histology. Sprague-Dawley rats were treated with vehicle (control); s.c. implanted human PTH (1-34) via osmotic pump (continuous PTH); or daily s.c. injection of PTH (pulsatile PTH). As expected, continuous PTH for 7 days induced tibial metaphysis histopathology resembling the second stage of osteitis fibrosa cystica or osteitis fibrosa, a generalized increase in osteoclastic bone resorption accompanied by fibroblasts and increased osteoblastic activity (data not shown). Pulsatile PTH dramatically increased bone formation without development of fibrosis.

PDGF-A is a candidate gene responsible for marrow fibrosis

The histological appearance of multilayer fibroblasts surrounding trabecular bone surfaces coupled with the apparent absence of PTH receptors on fibroblasts and the evidence of the receptors on osteoblasts^{8,9} suggest a paracrine communication by which PTH induces the release of one or more growth factor(s) by cells of the osteoblast

lineage, which induces fibrosis. To understand the etiopathogenesis, we therefore used cDNA microarrays to identify differentially expressed genes. Total cellular RNA was isolated from the same region of contralateral tibiae of rats on which histological measurements were performed. The RNA was hybridized with rat genechip microarrays containing 5531 genes, and data from rats treated with continuous PTH were compared to pulsatile PTH. Approximately 14 % of total genes measured were differentially expressed at least 2.5-fold. One of these was PDGF-A, a known mitogenic and chemotactic factor for fibroblasts. We next verified the gene microarray data using an RNase protection assay (Fig. 2a). Pulsatile PTH had no effect on steady-state mRNA levels for PDGF-A, whereas continuous PTH resulted in a 3.3-fold increase in expression of PDGF-A mRNA (Fig. 2b).

Trapidil decreases PTH-induced marrow fibrosis and osteoclastic resorption

Based upon the above findings, we postulated that agents that block PDGF-A binding to its cell-surface receptor are potential therapeutic agents for PTH-induced skeletal disorders. Triazolopyrimidine (trapidil) is a competitive inhibitor of PDGF receptor¹⁰. We examined the effect of trapidil on serum chemistry. Trapidil alone had no effect on serum calcium, phosphorus, magnesium or PTH level (Table 1). Continuous PTH induced hypercalcemia and increased serum PTH. Combination treatment of PTH and trapidil (PTH+trapidil) decreased serum calcium compared to PTH alone; however, the level was higher than in the controls.

We further investigated the effects of trapidil on PTH-induced skeletal disorders using bone histomorphometry. Continuous PTH stimulated cancellous bone formation

rate, whether expressed per bone surface, bone volume or tissue volume, due to an increase in osteoblast number as deduced by increased calcein labeled surface (Table 2). The fluorochrome-based analyses were confirmed by measurements showing that PTH increased osteoblast surface (Fig. 3a). Continuous PTH slightly increased mineral apposition rate, an index of osteoblast activity. Trapidil had no significant effect on measurements related to the PTH-induced increase in bone formation. As expected, continuous PTH increased osteoclast surface, an index of bone resorption (Fig. 3b, 4c) and induced extensive peritrabecular fibrosis (Fig. 3c, 4c). Trapidil decreased osteoclast perimeter and peritrabecular fibrosis induced by PTH by 73 and 63 %, respectively. Two-way ANOVA confirmed an interaction between PTH and trapidil on osteoclast and peritrabecular fibrotic perimeter, indicating that trapidil antagonized PTH but had no effect on normal rats. Interestingly, combination treatment with PTH and trapidil resulted in histological changes (Table 2 and Fig. 4d) similar to those previously observed following pulsatile PTH treatment, as shown by an increase in bone formation and osteoblast surface. These data indicate that trapidil, by antagonizing PDGF-A, reduces skeletal pathology induced by continuous infusion of PTH.

Discussion

In this study, we identify PDGF-A as a candidate signaling factor which contributes to skeletal pathology in an animal model for osteitis fibrosa cystica. Treatment of hyperparathyroid animals with trapidil provides a highly effective therapy for preventing the skeletal disorders, leading to a striking reduction of peritrabecular fibrosis and osteoclastic bone resorption.

PTH has incongruous effects on bone metabolism, either anabolic or catabolic, depending on the pattern of exposure to the hormone¹¹⁻¹³. Pulsatile PTH increases bone formation, whereas continuous PTH stimulates bone resorption and induces marrow fibrosis. The signaling pathway of PTH begins with its binding to the highly conserved PTH/PTH-related peptide receptor at the osteoblast surface. Upon binding to its receptor, early events are sufficient to induce anabolic response. However, late events associated with continuously elevated PTH lead to catabolic activity and bone disease. Therefore, understanding the differential gene expression of these two regimens is essential for determining what signals mediate the PTH-induced osteitis fibrosa cystica. Identification of genes induced by continuous versus pulsatile PTH was performed using cDNA microarray, a powerful tool for profiling gene expression. Using this approach, we show for the first time that PDGF-A mRNA becomes chronically elevated by continuous but not pulsatile PTH.

PDGF is a homo- or heterodimer polypeptide encoded by two distinct genes, PDGF-A and PDGF-B¹⁴. The PDGF-A and PDGF-B chains share 56% homology and join by disulfide bond to form three different dimers, AA, AB or BB^{15,16}. Cellular responses are mediated via two high-affinity receptor subunits, α and β . PDGF-A binds primarily to the α -receptor, whereas PDGF-B binds to the α - or β -receptors¹⁷. PDGF has a potent mitogenic and chemotactic property for mesenchymal cells. It plays a critical role in physiological repair mechanism and pathogenesis of proliferative diseases, including tumorigenesis, atherosclerosis, inflammatory disorders and fibrosis^{18,19}.

In skeletal tissues and isolated cells, the PDGF-A gene is expressed in normal osteoblasts, malignant skeletal cells and osteosarcoma cell lines^{14,20}. The prevalence of the

PDGF- α receptor on osteoblasts but not osteoclasts at remodeling sites suggests a local role of PDGF-A in the regulation of osteogenesis²¹. Preliminary studies, using ³H-thymidine radioautography to determine the origin of the peritrabecular fibroblasts induced by continuous infusion of PTH, indicated extensive proliferation of the fibroblasts lining bone surfaces (unpublished data). This finding as well as an increase in PDGF-A mRNA expression in the present study strongly suggest a role for PDGF-A in the recruitment and expansion of fibroblast populations by PTH.

From a clinical perspective, osteitis fibrosa cystica is considered one of the most problematic skeletal diseases. The diagnosis is straightforward when the disease has reached an advanced stage but the pathology is extremely difficult to treat. When bone pain fails to resolve, the patients typically undergo parathyroidectomy. The discovery of PDGF-A as a candidate gene in mediating bone damage in chronic hyperparathyroid animals leads to a novel therapeutic strategy in which PDGF-A and its receptors become important targets of the treatment.

Trapidil, originally developed as a vasodilator and anti-platelet agent, has proven to be clinically effective in the treatment of coronary heart disease in Japan^{22,23}. It acts as an inhibitor of phosphodiesterase, thromboxane A₂ and PDGF. It was suggested that trapidil inhibited the stimulating action of PDGF via competitive binding with PDGF receptor on the target cells¹⁰.

Although antiproliferative effects of trapidil have been described, trapidil at the dosage of 50 mg/kg body weight, which was higher than that used in this study, had no significant cytotoxic effect²². We also demonstrated that trapidil alone had no effect on body weight (data not shown), serum chemistry and bone histomorphometry. Continuous

infusion of PTH was shown in this and a previous study⁷ to increase serum PTH level. In addition, continuous PTH resulted in hypercalcemia. PTH acts to increase serum calcium by (i) stimulation of $1,25\text{ (OH)}_2\text{D}_3$ production, leading to an increase in intestinal absorption of calcium, (ii) augmentation of renal tubular calcium reabsorption, and (iii) stimulation of osteoclastic bone resorption²⁴. The action of PTH on osteoclastic development and maturation is through stimulation of receptor activator of nuclear factor kappaB ligand (RANKL) which anchors to the cell surface of osteoblasts²⁵. It remains unclear whether PTH can stimulate RANKL synthesis directly or indirectly through secretion of cytokines which in turn regulate RANKL synthesis.

Treatment of animals with trapidil did not alter the effect of continuous PTH administration on serum PTH level which demonstrates that it did not act by accelerating the metabolism of the hormone. Trepidil, however, did decrease serum calcium in PTH-treated rats. This reduction of serum calcium was likely due to an inhibitory effect of trapidil on osteoclastic resorption, as shown by a decrease in osteoclast surface. The effects of trapidil on intestinal calcium absorption and renal tubular reabsorption are unknown. All three isoforms of PDGF were shown to induce proliferation of osteoblastic cells in culture (PDGF-BB > -AB > -AA)^{26,27}. Although PDGF increases the number of cells capable of synthesizing collagen, it inhibits matrix apposition in intact calvariae²⁸. Trepidil failed to antagonize the effect of continuous PTH on osteoblast number and activity, indicating a minor role of PDGF in the initial stimulation of bone formation by PTH. However, trapidil dramatically suppressed osteoclast surface. PDGF-BB was reported to stimulate bone resorption, possibly by increasing the expression of interleukin-6 (IL-6)²⁹. PDGF-AA was also shown to increase IL-6³⁰ and as a consequence

could increase bone resorption. Additionally, PTH also enhances bone resorption by increasing IL-6 production³¹. Recently, fibroblastic cells have been reported to express RANKL³². The factors which augment RANKL and diminish RANKL decoy receptor osteoprotegerin (OPG) mRNA levels in these cells cause osteoclastogenesis. The mechanism for the remarkable decrease in osteoclast recruitment is unclear but may be due to the antagonistic activity of trapidil on PDGF and marrow fibrosis.

Continuous infusion of PTH resulted in tibial metaphysis histological changes similar to a previous report, including multilayers of fibroblast cells lining trabeculae⁷. Trepidil was found to inhibit Balb/c 3T3 fibroblast proliferation induced by PDGF (ref. 33) and we demonstrated that trapidil markedly reduced peritrabecular fibrosis. Trepidil was also reported to strongly decrease gene expression of PDGF- α and - β receptor and to moderately suppress mRNA level of PDGF-A and -B in injured rat arteries²³. Studies in humans have demonstrated that skeletal pathology is reversible after parathyroidectomy².

In conclusion, PDGF-A serves as a critical factor in initiation of skeletal pathology in hyperparathyroidism. Trepidil, a PDGF antagonist, suppressed the development of peritrabecular fibrosis and osteoclastic bone resorption while allowing stimulation of bone formation to continue. The beneficial effects of trapidil on bone with no adverse effects at the dosage tested in this study suggest a use of PDGF antagonists in chronic renal failure. Whether or not trapidil could reduce established marrow fibrosis requires further investigation.

Methods

Induction of osteitis fibrosa cystica. Female Sprague-Dawley rats (3-month-old, Harlan Sprague-Dawley, Inc., Indianapolis, IN) were randomly divided into 3 groups with 5 animals per group. One group was given daily s.c. injection with 80 µg/kg BW/day human PTH (1-34) (hPTH) for 7 days. The hPTH was dissolved in a solution containing 150 mM NaCl, 1 mM HCl and 2% heat-inactivated rat serum. The two other groups received s.c. implanted osmotic pumps (Alza Corp., Mountainview, CA) which delivered vehicle or 40 µg/kg BW/day hPTH at the rate of 1 µl/hr for 7 days. On day 8, animals were anesthetized with ketamine (50 mg/kg BW) : xylazine HCl (5 mg/kg BW) and sacrificed by decapitation, and both tibiae were removed. Right tibiae were fixed by immersion in 70% ethanol and processed for bone histology in order to verify an appearance of peritrabecular fibrosis. Left tibiae were frozen in liquid N₂, stored frozen at -80 °C until processed for RNA isolation.

Isolation of RNA. The frozen proximal tibial metaphyses were individually homogenized in guanidine isothiocyanate using a Spex freezer mill (Spex Industries, Inc., Edison, NJ). Total RNA was extracted and isolated using a modified organic solvent method and the RNA yields were determined spectrophotometrically at 260 nm (ref. 34).

cDNA microarray analysis. Rat genefilter microarrays (GF 300), consisting of 5531 genes were purchased from Research Genetics (Huntsville, AL). cDNA probes were generated from 1 µg total RNA isolated from tibial metaphyses from each group of animals by reverse transcription (Superscript II, Life Technologies, Rockville, MD). First-strand cDNA probes were primed by addition of Oligo dT and labeled with [α -³³P]dCTP (ICN Radiochemicals, Costa Mesa, CA). The probes were subsequently

purified by passage through Sephadex G-50 DNA Grade Column (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Hybridization was carried out as recommended by manufacturer's protocol. After the hybridization, the array was washed and wrapped with plastic wrap before placing it in a phosphor imaging cassette containing Cyclone Storage Phosphor Screen (Packard, Downers Groves, IL) for 24 hours. The array was scanned and the image was analyzed using Pathways 2.01 software to compare the signal intensities of spots.

RNase protection assay. Steady-state mRNA levels for PDGF-A were determined using an RNase protection assay according to the manufacturer's protocol (Pharmingen, San Diego, CA). Quantitation of protected RNA fragments was performed by PhosphoImager analysis and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal structural protein L32.

Treatment protocol. Three-month-old female Sprague-Dawley rats were divided into 4 groups [Vehicle ($n = 9$), trapidil ($n = 10$), PTH ($n = 8$) and PTH+trapidil ($n = 10$)]. The animals were implanted s.c with osmotic pumps containing 1 week's dose of either vehicle or 40 $\mu\text{g/kg BW/day}$ hPTH. They also received daily s.c. injections of vehicle or 40 mg/kg BW/day trapidil (Rodleben Pharma GmbH, Rodleben, Germany) for 8 days. This dosage was estimated on the basis of an effective inhibitory effect of trapidil on several types of cells in the rat^{22,35,36}. Fluorochrome labels (20 mg/kg BW , Sigma Chemical Co., St. Louis, MO) were injected at the base of the tail on day 0 (tetracycline) and day 6 (calcein). At the end of experiment (day 8), the rats were anesthetized and blood was collected by cardiac puncture for determination of serum chemistry and PTH

levels. They were sacrificed by cervical dislocation and tibiae were removed and fixed in 70% ethanol for bone histomorphometry.

Serum chemistry and PTH. Total serum calcium, phosphate, and magnesium were measured by Central Clinical Laboratory Research at the Mayo Clinic using an automated procedure. Serum PTH was measured using an immunoradiometric assay for rat PTH (Immunotopics International, LLC, San Clemente, CA) which has approximately 100% cross-reactivity to human PTH.

Bone histomorphometry. The proximal metaphyses were dehydrated in a graded series of ethanol, infiltrated and embedded in methylmethacrylate (Fisher Scientific, Fair Lawn, NJ). Tissue sections were cut at 5 μm thickness (Reichert-Jung Model 2065 Microtome, Heidelberg, Germany) and mounted unstained for dynamic cancellous bone measurements. Consecutive sections were stained with toluidine blue for bone cell and peritrabecular fibrosis measurements and Goldner's method for photomicrographs. A standard sampling site of 2.8 mm^2 was established in the secondary spongiosa of the metaphysis 1.5 mm distal to the growth plate. All histomorphometric measurements were carried out with an Osteomeasure image analysis system (OsteoMetrics, Atlanta, GA) coupled to a photomicroscope and personal computer and parameters were calculated according to the standardized nomenclature³⁷. Bone volume was defined as the percentage of tissue volume consisting of cancellous bone. Tetracycline and calcein labels were the length of bone perimeter labeled with fluorochrome expressed as a percentage of bone perimeter. Mineral apposition rate was the average width between tetracycline and calcein label divided by interlabel time of 6 days. Bone formation rate was calculated as the product of mineral apposition rate and the calcein labeled perimeter,

expressed per bone surface, bone volume or tissue volume. Osteoblast surface, identified as a palisade of large basophilic cuboidal cells directly lining the osteoid, was expressed as a percent of bone perimeter. Osteoclast surface was determined as the bone perimeter lined by multinucleated cells regardless of the presence of erosion. Fibrotic perimeter was the bone perimeter lined by multilayers of fibroblasts.

Statistic analysis. Multiple group comparisons were determined using one-way analysis of variance (one-way ANOVA) with statistical significance at $P < 0.05$. Differences between pairs of groups were compared by the Fisher's protected least significant difference post-hoc test. Two-way analysis of variance (two-way ANOVA) was performed to determine significant effects of PTH and trapidil or interaction between PTH and trapidil.

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Figure Legends.

Fig. 1 Osteitis fibrosa cystica in renal osteodystrophy patients with secondary hyperparathyroidism. Microscopic appearance of iliac crest biopsy with Goldner's stain exhibiting fibrosis surrounding trabeculae (black arrows) with excessive osteoid formation (bright red regions on trabecular surfaces) in mild osteitis fibrosa (*a*), and showing numerous osteoclasts (white arrows) and extensive marrow fibrosis (black arrows point to regions) in severe osteitis fibrosa cystica (*b*).

Fig. 2 Continuous but not pulsatile PTH increases mRNA levels for PDGF-A. Rats were treated with vehicle, daily s.c. injection of hPTH (1-34) (pulsatile PTH) or s.c. implanted hPTH (1-34) osmotic pump (continuous PTH) for 7 days. Tibial metaphyses were removed for total RNA extraction. *a*, Representative RNase Protection Assay for PDGF-A and housekeeping genes, L32 and GAPDH. *b*, Results are expressed in arbitrary densitometric units normalized for the expression of L32 in each group. Continuous PTH significantly increased PDGF-A either expressed per L32 or GAPDH (data not shown). Each bar represents mean \pm s.e.m. ($n=5$). ^a, $P < 0.001$ compared with vehicle, ^b, $P < 0.01$ compared with trapidil.

Fig. 3 Effects of trapidil, PTH and combination of PTH and trapidil on bone cells and fibroblasts. Rats were treated as described in Table 1. Five-micron-thick tibial longitudinal sections were stained with toluidine blue for measuring: *a*, Osteoblast surface per bone surface (Ob.S/BS). *b*, Osteoclast surface per bone surface (Oc.S/BS). *c*, Fibroblasts surrounding trabecular surface (fibrotic perimeter). Two-way ANOVA

indicated significant effects of PTH on Ob.S/BS whereas there were significant effects of PTH, trapidil or interaction between PTH and trapidil on Oc.S/BS and fibrotic perimeter. Each bar represents mean \pm s.e.m. ($n = 8-10$). ^a, $P < 0.05$ compared with vehicle, ^b, $P < 0.05$ compared with trapidil, ^c, $P < 0.05$ compared with PTH.

Fig. 4 Inhibitory effects of trapidil on peritrabecular fibrosis induced by continuous PTH. Rats were treated as described in Table 1. Photomicrograph of Goldner's stain from tibial metaphyses in rats treated with vehicle (**a**), trapidil (**b**), PTH (**c**), PTH and trapidil (**d**). Note numerous fibroblasts (black arrows) and osteoclasts (white arrows) in **c** and osteoblasts (black arrows) in **d**.

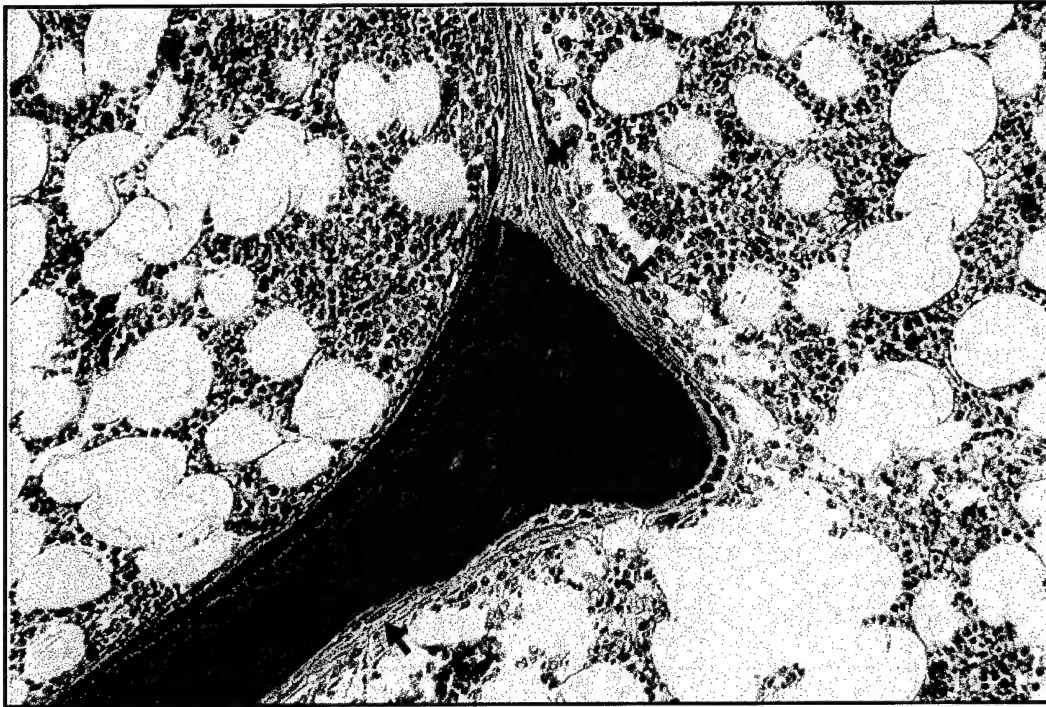


Fig. 1a

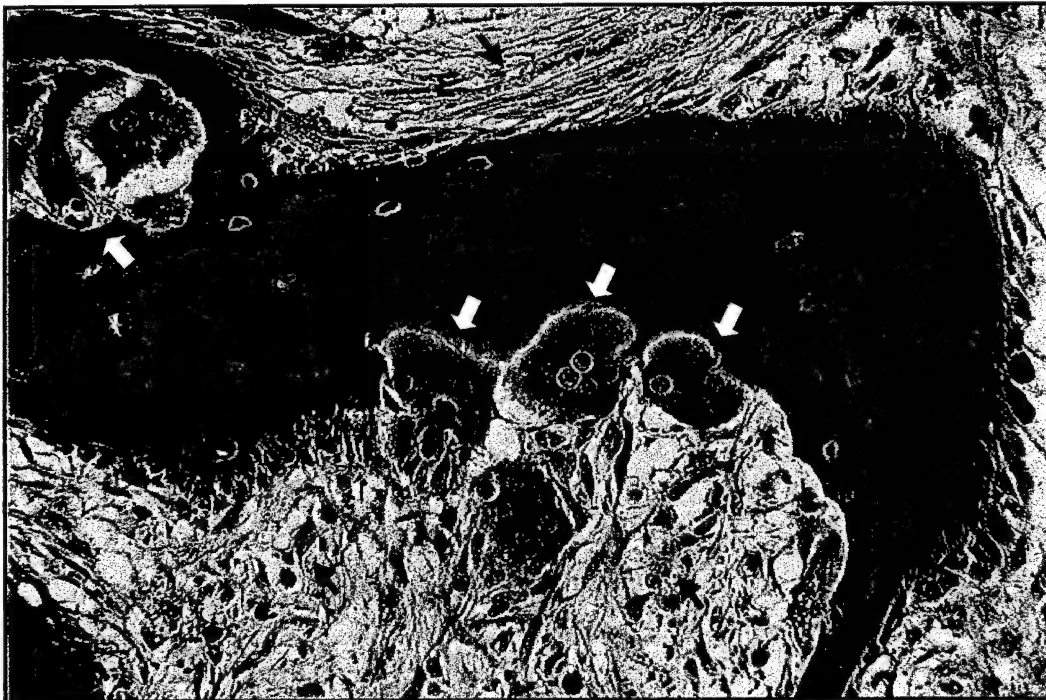


Fig. 1b

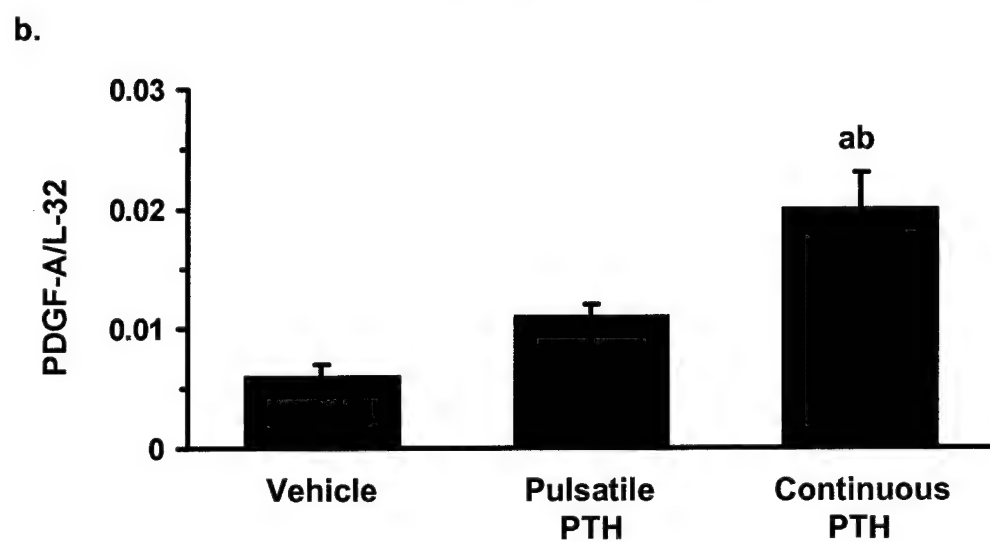
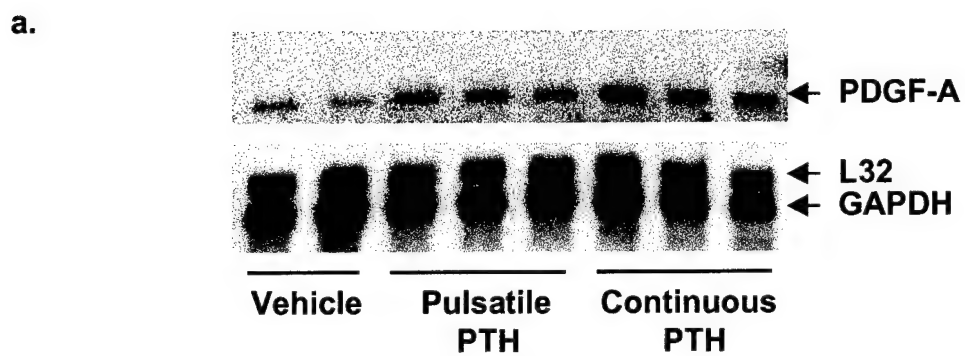


Fig. 2

Table 1 Continuous PTH induced hypercalcemia and elevated serum PTH.

Parameters	Vehicle (n = 9)	Trapidil (n = 10)	PTH (n = 8)	PTH+Trapidil (n = 10)	Two-way ANOVA		
					PTH	Trapidil	Interaction
Calcium (mg/dl)	10.26 ± 0.07	10.10 ± 0.05	11.65 ± 0.28 ^{ab}	10.94 ± 0.32 ^{abc}	P < 0.001	P < 0.05	NS
Phosphorus (mg/dl)	7.89 ± 0.27	7.33 ± 0.29	7.95 ± 0.48	7.46 ± 0.29	NS	NS	NS
Magnesium (mg/dl)	2.34 ± 0.05	2.40 ± 0.05	2.49 ± 0.04	2.46 ± 0.07	NS	NS	NS
PTH (pg/ml)	43.29 ± 7.58	51.98 ± 8.21	98.27 ± 11.12 ^{ab}	118.49 ± 34.16 ^{ab}	P < 0.05	NS	NS

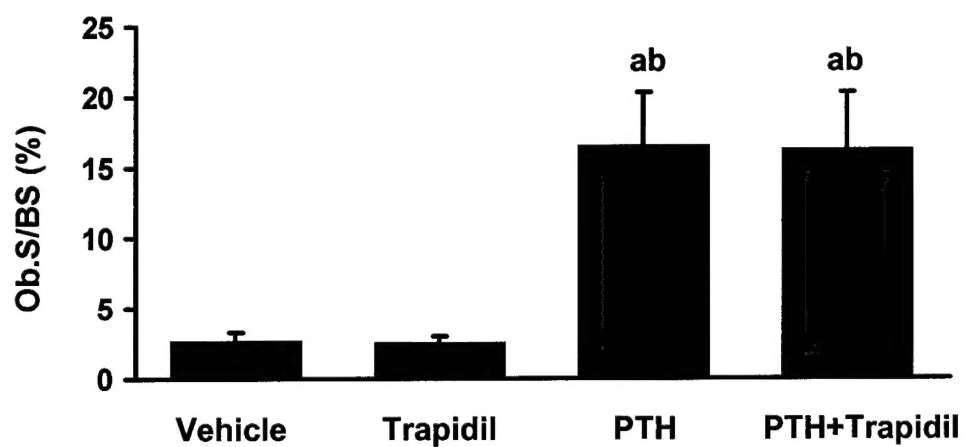
Rats were s.c. implanted with 7-day-release osmotic pump containing vehicle in vehicle and trapidil groups or 40 µg/kg/day hPTH (1-34) in PTH and PTH+trapidil groups. They also received daily s.c. injection of vehicle in vehicle and PTH groups or 40 mg/kg/day trapidil in trapidil and PTH+trapidil groups. Data represent mean ± s.e.m. ^a, P < 0.05 compared with vehicle, ^b, P < 0.05 compared with trapidil, ^c, P < 0.05 compared with PTH.

Table 2 Continuous PTH stimulates cancellous bone formation rate.

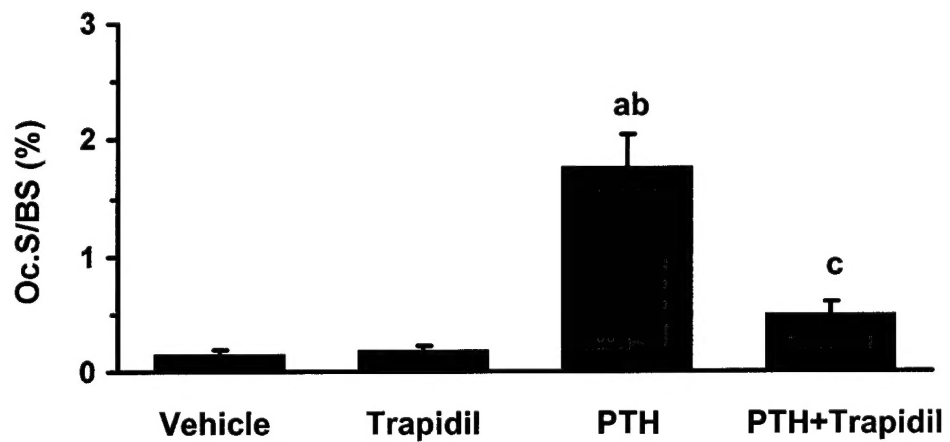
Parameters	Vehicle (n=9)	Trapidil (n=10)	PTH (n=8)	PTH+Trapidil (n=10)	Two-way ANOVA		
					PTH	Trapidil	Interaction
BV/TV (%)	24.89 ± 0.94	24.83 ± 0.87	27.25 ± 1.14	23.79 ± 0.86	NS	NS	NS
Tetracycline label (%)	7.86 ± 0.94	9.65 ± 1.76	5.65 ± 2.48	6.91 ± 1.71	NS	NS	NS
Calcine label (%)	18.63 ± 2.22	18.32 ± 1.39	48.08 ± 5.35 ^{ab}	43.24 ± 4.42 ^{ab}	P < 0.001	NS	NS
MAR (µm/day)	1.09 ± 0.05	1.06 ± 0.04	1.29 ± 0.08	1.18 ± 0.07	P < 0.05	NS	NS
BFR/BS (µm ³ /µm ² /day)	0.29 ± 0.03	0.30 ± 0.03	0.71 ± 0.10 ^{ab}	0.59 ± 0.06 ^{ab}	P < 0.001	NS	NS
BFR/BV (%)	1.04 ± 0.11	0.99 ± 0.08	2.30 ± 0.31 ^{ab}	2.06 ± 0.18 ^{ab}	P < 0.001	NS	NS
BFR/TV (%)	0.26 ± 0.03	0.25 ± 0.02	0.63 ± 0.09 ^{ab}	0.49 ± 0.05 ^{ab}	P < 0.001	NS	NS

Rats were treated as described in Table 1. They were perivascular tail injected with 20 mg/kg tetracycline on the first day of experiment and calcein on the second day before sacrifice to label bone. Undemineralized 5 µm thick sections from tibial metaphases were used to determine cancellous bone histomorphometry. Data represent mean ± s.e.m.^a, P < 0.05 compared with vehicle,^b P < 0.05 compared with trapidil,^c P < 0.05 compared with PTH. Abbreviation: bone volume per tissue volume (BV/TV), mineral apposition rate (MAR), bone formation rate (BFR) expressed per bone surface (BS), bone volume (BV) or tissue volume (TV).

a.



b.



c.

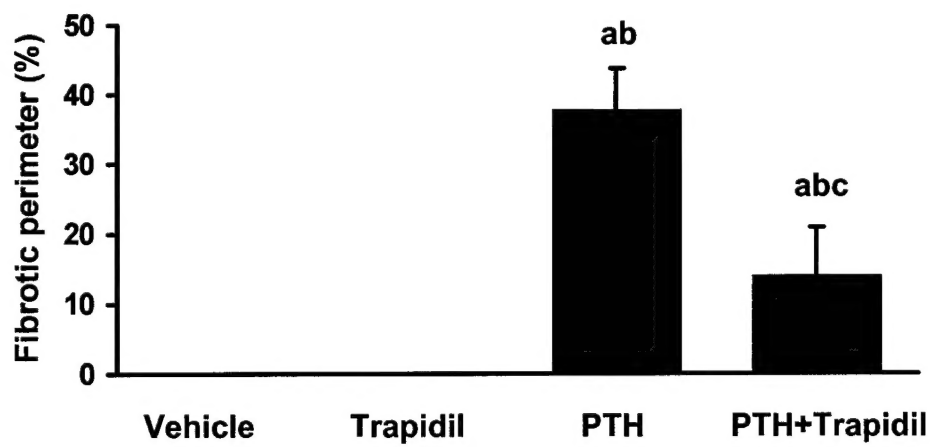


Fig. 3

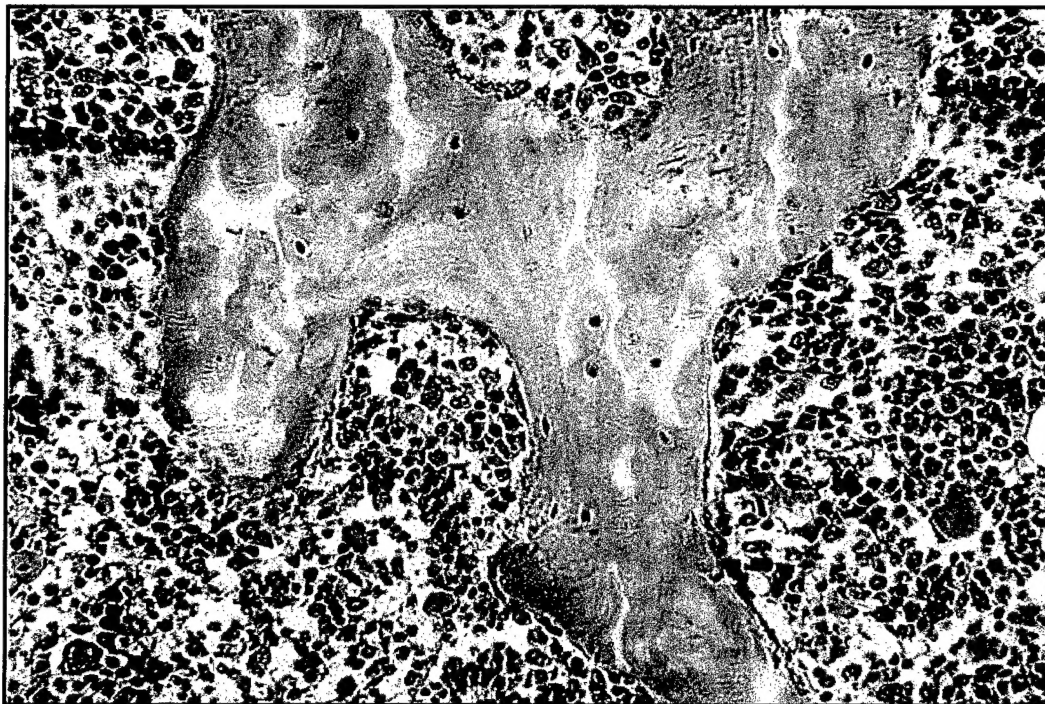


Fig. 4a

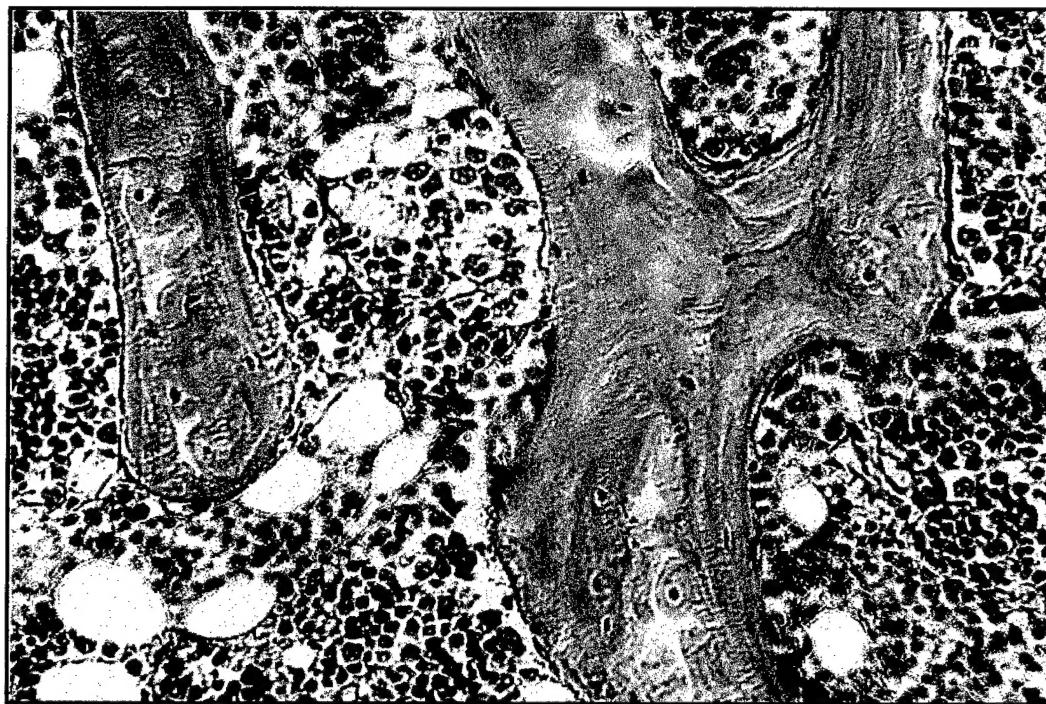


Fig. 4b

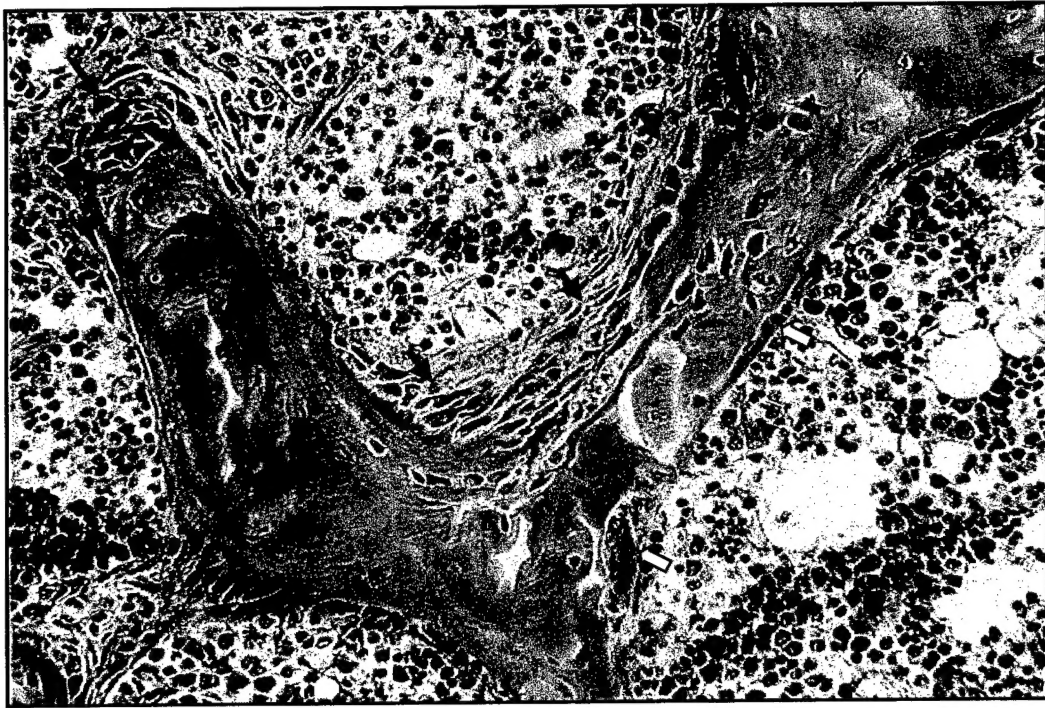


Fig. 4c



Fig. 4d